DNA Methylation Analysis in Childhood Acute Lymphoblastic Leukemia

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Thesis Abstract

PART I. Identification of Novel Aberrantly Methylated CpG Islands in Childhood ALL

Aberrant promoter hypermethylation is recognized as a hallmark of human malignancies. This aberration leads to transcriptional inactivation of cancer-related genes, and thus malignant transformation. Currently, candidate gene approach is commonly used for the investigation of DNA methylation involvement in various human cancers. By this approach, it was demonstrated that aberrant DNA hypermethylation plays a significant role in leukemogenesis.

To identify novel cancer-related genes in childhood acute lymphoblastic leukemia (ALL), we carried out a genome-wide screening for aberrantly methylated CpG islands by studying and comparing the methylation patterns of patient leukemic samples (N=8; 4 pre-B ALL, 2 common-ALL and 2 T-cell ALL) and normal peripheral blood (N=4) using methylation-sensitive arbitrarily primed polymerase chain reaction (MS-AP PCR). We found 40 genomic regions showing differential DNA methylation patterns between the leukemic and normal samples. DNA sequencing and BLAT search revealed 10 of them were located either at or near by the 5' putative promoter regions with dense CpG islands of known genes (N=9) and

uncharacterized gene (N=1). Among these candidates, *Fibrillin 2* (*FBN2*), located on chromosome 5q23.1, was selected for further study as it demonstrated concurrent aberrant promoter hypermethylation (697, CCRF-CEM, MOLT-3, REH and Rs4;11) and transcriptional silencing (CCRF-CEM, MOLT-3 and Rs4;1) or down-regulation (697) in all 5 ALL cell lines except REH, which showed *FBN2* expression despite presence of promoter methylation. Using bisulfite sequencing analysis, we demonstrated a strong correlation between *FBN2* methylation density and the gene expression in ALL cell lines. High methylation density was found in the ALL cell lines with loss or reduced gene expression, whereas REH with normal expression revealed low methylation density.

PART II. Methylation Studies of *FBN2* in ALL Cell Lines and Clinical Samples of childhood ALL

We performed demethylation studies and demonstrated that treatment by 5-aza-2'-deoxycytidine on the *FBN2* non-expressing or down-regulated cell lines induced a dose-dependent reactivation (CCRF-CEM, MOLT-3 and Rs4;11) or up-regulation (697) of *FBN2* expression, thus confirming the role of promoter methylation in transcriptional silencing of *FBN2*.

FBN2 is a large modular extracellular matrix glycoprotein that functions as a

beta-1 integrin ligand for cell adhesion. It is possible that it may play a role in the cellular interactions that are important in ALL biology in the bone marrow microenvironment. Next, we studied the involvement of *FBN2* hypermethylation in clinical leukemic samples and found 69 % (44/64) of childhood ALL samples revealed this aberration (B lineage ALL: 66 % [38/58]; T lineage ALL: 100 % [6/6]). In addition, in these leukemic samples, we demonstrated concordant findings of loss or down-regulation of *FBN2* mRNA by semi-quantitative RT-PCR and hypermethylated status by COBRA analysis.

Taken together, we successfully demonstrated the feasibility of MS-AP PCR in the identification of novel DNA methylation-regulated genes in leukemogenesis and our results suggested DNA hypermethylation associated *FBN2* silencing may be potentially involved in the pathogenesis of childhood ALL. In view of the high frequency of *FBN2* promoter hypermethylation, this aberration may also prove to be a useful tumor marker for childhood ALL in future.

論文摘要

一、與兒童急性淋巴白血病相關之異常甲基化 CpG 島的發現

啓動子異常甲基化被認爲是人類癌症的重要標誌，這種異常甲基化是導致人類抑癌基因表達缺失的主要機制之一。現時，與異常甲基化相關的基因在不同癌症的角色只是利用已知的抑癌基因甲基化鑒定調查爲主。這種方法的研究結果顯示，異常甲基化在血癌的形成中發揮著重要的作用。

爲了探討與兒童急性淋巴白血病相關的新基因，我們利用兒童急性淋巴白血病的臨床標本 (四個 pre-B ALL，兩個 common ALL 和兩個 T-ALL) 及四個正常周邊血液標本執行了一次寬查性的甲基化敏感性隨機引物聚合酵素連鎖反應檢查，從而發現異常甲基化的 CpG 區域。利用兒童急性淋巴白血病及正常周邊血液標本的甲基化圖譜對照，我們發現了四十個不同的甲基化的區域。分析顯示出其中的十個區域是位於或靠近 5' 啓動子區域的 CpG 島，在這十個片段中，九個是已知的基因，一個是新基因。我們進一步根據 CpG 島甲基化和訊息核糖核甘酸的表達度的關係，檢查了這十個基因在五株急性淋巴白血病細胞株 (697，CCRF-CEM，MOLT3，REH，Rs4;11) 中的異常甲基化及訊息核糖核甘酸的表達度。*Fibrillin 2* (*FBN2*) 啓動子的異常甲基化在五株急性淋巴白血病細胞株中得到證實，然而在 CCRF-CEM，MOLT3 和 Rs4;11 則發現訊息核糖核甘酸的抑制，697 則發現訊息核糖核甘酸的下調。我們利用亞硫酸鹽序列分析，COBRA 和半定量逆轉錄聚合酶鏈反應證實了 *FBN2* 在急性淋巴白血病細胞株中高度甲基化和基因表達減少的關係 (697，CCRF-CEM，MOLT3，Rs4;11)。亞硫酸鹽序列分析的結果亦揭示了 *FBN2* 在 REH 的正表達是由低甲基化密度引致。根據這些篩選標準，顯示了 *FBN2* 是可能的候選基因。

二、***FBN2*** 甲基化分析

利用去甲基化藥物 (5-aza-2'deoxycytidine) 處理，可以誘發 *FBN2* 完全甲基化和訊息核糖核甘酸抑制 (CCRF-CEM，MOLT3，Rs4;11) 或下調 (697) 的細胞株作重新表達。這個結果證實了 *FBN2* 訊息核糖核甘酸的抑制或下調是由啓動子異常甲基化機制所控制。因此，我們進一步探討了 *FBN2* 的異常甲基化在兒童急性淋巴白血病的臨床意義。分析顯示了 69 % (44/64) 的兒童急性淋巴白血病病人帶有 *FBN2* 的異常甲基化，而 *FBN2* 的訊息核糖核甘酸的表達亦與臨床標本甲基化的情度吻合。

總括而言，我們成功地使用了寬查性的甲基化敏感性隨機引物聚合酵素連鎖反應的方法探討了異常甲基化調節與兒童急性淋巴白血病相關的基因。結果顯示 *FBN2* 的異常甲基化是調控其訊息核糖核甘酸表達度的重要機制之一。這個研究揭示了 *FBN2* 訊息核糖核甘酸的抑制可能與兒童急性淋巴白血病的形成機制有關，更引證了 *FBN2* 的異常甲基化作爲一個兒童急性淋巴白血病的腫瘤標誌的可能性。

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Abbreviations

5-Aza-dCR	5-aza-2'-deoxycytidine
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
Apaf-1	Apoptotic peptidase activating factor
APC	Adenomatous polyposis coli
APS	Adaptor protein with pleckstrin homology and src homology 2 domains
ASPP1	Protein phosphatase 1
ATCC	American Type Culture Collection, Manassas, VA, USA
ATXN2	Ataxin 2
BFM95	German Berlin-Frankfurt-Muenster 95
BM	Bone marrow
BRCA1	Breast cancer 1
CALC1	Calcitonin
CDK	Cyclin-dependent kinase
CDKN2A/p16	Cyclin-dependent kinase 2A
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
CNS	Central nervous system
COBRA	Combined bisulfite restriction analysis
DAPK	Death-associated protein kinase 1
DFS	Disease-free survival
Dkk-3	Dickkopfs-3
DMH	Differential methylation hybridization
DMNT	DNA-methyltransferases
DMSO	Dimethyl sulfoxide
DMSZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
Early pre-B ALL	Early precursor-B cell ALL

ECM	Extracelluar matrix
EDNRB	Endothelin receptor B
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ER	Estrogen receptor
FAB classification	French-American-British classification
FBN2	Fibrillin 2
FBS	Fetal bovine serum
FHIT	Human fragile histidine triad gene
FISH	Fluorescence in situ hybridization
FM	Full methylation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSTP1	Glutathione S-transferase P1
GTE	(Solution of 50mM glucose, 25nM Tris-Cl (pH 8.0), 10mM EDTA)
H3-K9	Histone H3 lysine 9
HAT	Histoneacetylase
HDAC	Histonedeacetylase
HSC	Hematopoietic stem cells
IL-12	Interleukin-12
LATS1	Large tumor suppressor, homolog 1
LOH	Loss of heterozygosity
LSC	Leukemic stem cell
MBD	Methyl-CpG binding domain
MC	Mononuclear cells
MCA-RDA	Methylated CpG island amplification coupled with representational difference analysis
MDS	Myelodysplastic syndrome
MeCP	Methyl cytosine binding proteins
MGMT	O^6-Methylguanine DNA methyltransferase
min(s)	Minute(s)
MLH1	mutL homolog 1
MLL	Mixed-lineage leukemia
MRD	Minimal residual disease
mRNA	Messager RNA
MS-AP PCR	Methylation sensitive arbitrarily primed polymerase chain reaction
MS-PCR	Methylation-sensitive PCR

MS-RDA	Methylation-sensitive representational difference analysis
MS-RF	Methylation-sensitive restriction fingerprinting
MS-SNuPE	Methylation-sensitive single nucleotide primer extension
MYF3	Myogenic differentiation 1 gene
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
NES1	Normal epithelial cell-specific 1 gene
NK cell	Natural killer cell
NNAT	Human neuronatin
NSCLC	Non-small cell lung cancer
OS	Overall survival
PAX6	Paired box gene 6
PB	Peripheral blood
PBS	Phosphate buffer saline
PM	Partial methylation
PMP24	Peroxisomal membrane protein 24kDa
PTAG	Pituitary tumor apoptosis gene
PTEN	Phosphatase and tensin homolog
RANBP1	RAN binding protein 1
Rb	Retinoblastoma tumor suppressor gene
RGD	Arginyl-glycyl-aspartyl
RLGS	Restriction landmark genomic scanning
RNA	Ribonucleic acid
Pre-B ALL	Precursor-B ALL
RT	Room temperature
RT-PCR	Reverse transcription-PCR
s	Second(s)
SAM	S-adenosylmethionine
SDS	Sodium dodecylsulfate
SEER	Surveillance Epidemiology and End Results
SERPINB5	Maspin
SKY	Spectral karyotyping
SNRPN	Human small nuclear ribonucleoprotein polypeptide N
TBE buffer	(44.5mM Tris-base, 49mM boric acid, 1mM EDTA)
TGF	Transforming growth factor
THBS1	Thrombospondin 1
THBS2	Thrombospondin 2
TMEFF2	Transmembrane protein with EGF-like and two

	follistatin-like domains 2
TIMP3	Metalloproteinase-3
Tris-EDTA	Tris-ethylenediamine tetraacetic actic
WBC	White blood cell
WHO	World Health Organization
ZBED4	Zinc finger, BED-type containing 4
ZHX2	Zinc fingers and homeoboxes 2

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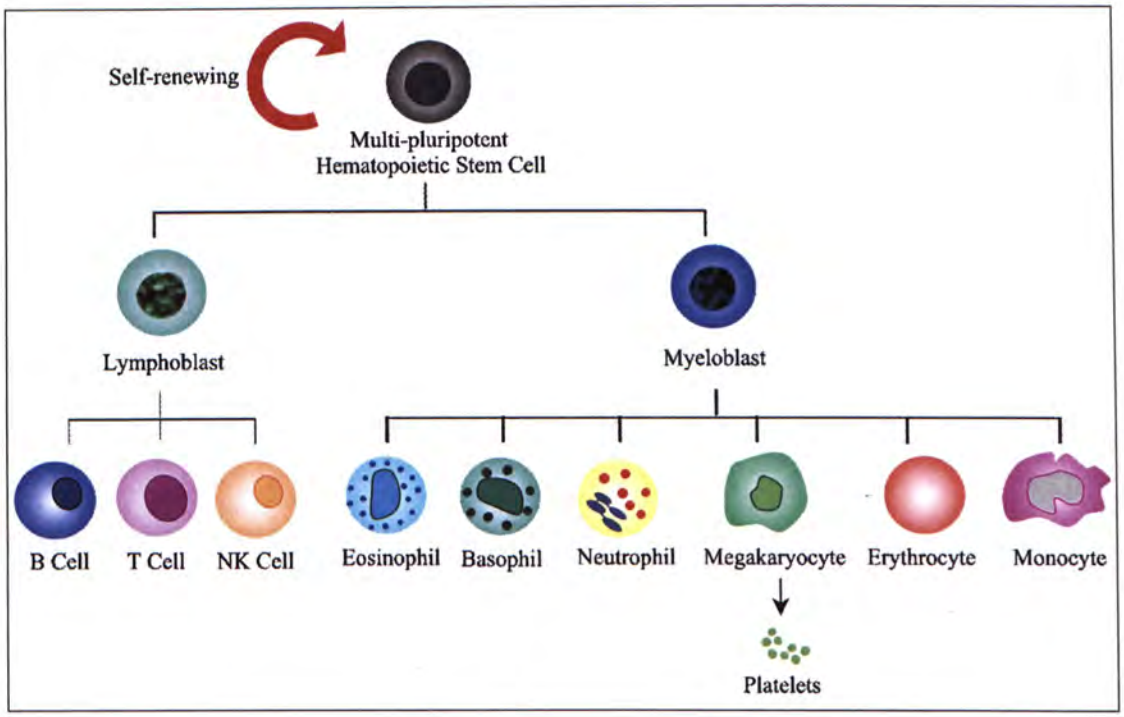
Chapter 1 Introduction

1.1. Normal Hematopoiesis

Hematopoiesis is the process of blood cell development that occurs in bone marrow (BM) of adults and children after birth. The continuous production of blood cells during the entire life span of an individual depends on a type of rare pluripotential cell, hematopoietic stem cell (HSC), whose abundance is tightly regulated by several processes, including self-renewal, proliferation, lineage specification, differentiation, maturation and apoptosis (Rafii *et al.*, 1997). Through the intrinsic cellular programming, cytokine signaling and interaction between adhesive molecules with the micro-environmental stroma, primitive HSC vigorously divides and differentiates into various types of blood cells, which, however, can be generalized into two major descendents, the myeloid and lymphoid lineages (Hoffbrand *et al.*, 2001). In particular, the myeloid lineage includes eosinophils, basophils, neutrophils, megakaryocytes, erythrocytes and monocytes. The lymphoid lineage, on the other hand, includes B lymphocytes, T lymphocytes and natural killer (NK) cells (Figure 1.1) (Orkin, 1995).

Figure 1. 1 *A schematic diagram of normal hematopoiesis.*

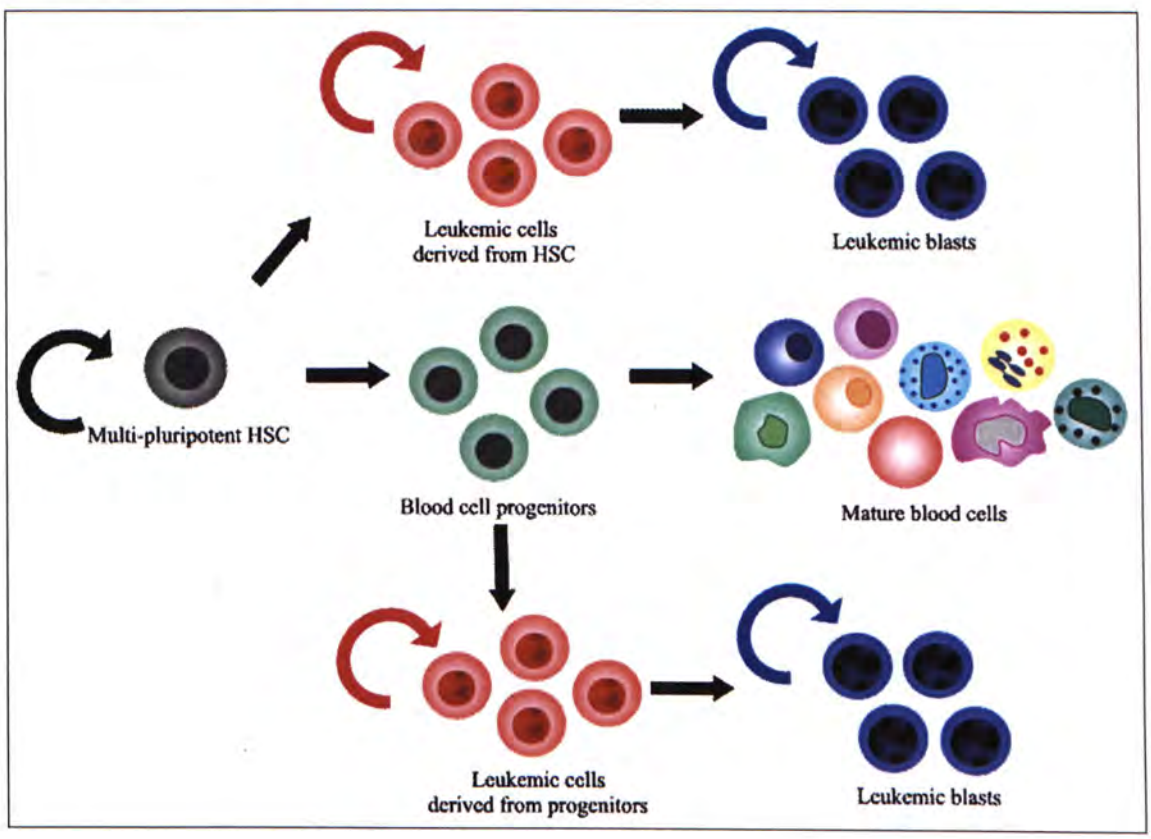


1.2. Hematological Malignancy and the Aberrant Development of Blood Cells

Hematological malignancy arises when the normal regulatory processes of hematopoiesis lose its control, accumulating aberrant blood cells in bone marrow and circulation (Downing and Shannon, 2002). The prevalence of hematological malignancies is estimated to 5 % - 8 % in adult cancers and over 50 % in childhood cancers (American Cancer Society, 2005). Previous report has demonstrated that certain unknown factors induced the transformation of HSC into rare leukemic stem cells (LSC) that render the maintenance and acquisition of uncontrolled hematopoiesis and therefore hematological malignancy (Hoffbrand *et al.*, 2001). More recent report

has suggested that the pluripoitency of HSC enables the cell to possess unlimited self-renewal property and to lengthily persist for the accumulation of aberrant genetic changes, allowing the derivation of LSC from HSC (Bonnet, 2005). Besides, the common developmental pathways of HSC and LSC may also be one of the factors for such unknown cause of transformation (Huntly and Gilliland, 2005) (Figure 1.2).

Figure 1. 2 *Origins of leukemic transformation events.*

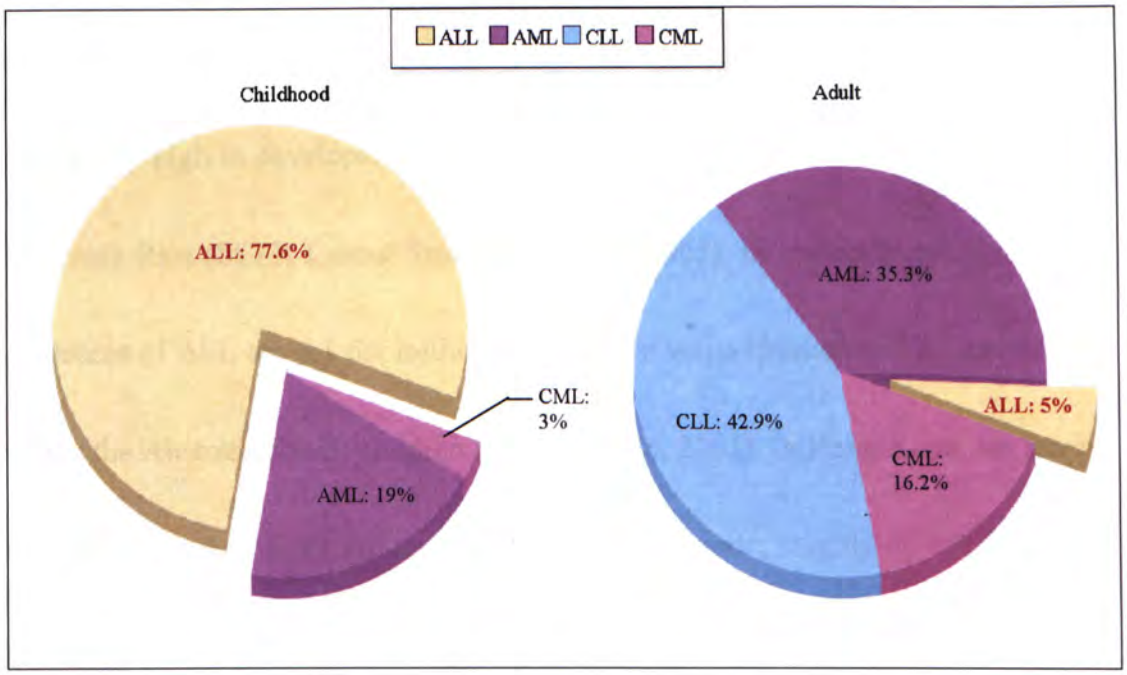


1.3. Leukemia and Its Classification

Leukemia, respectively affect 31 % and 25 % of cancer patients aged under 15 and 20 years old, is the commonest type of hematological malignancy that observed in

children (SEER Cancer Statistics Review, 2005; Pui and Evans, 1998; Pui, 2000) (Figure 1.3). It can be divided into acute and chronic types according to the degree of maturity of the predominant leukemic cell type. Specifically, acute leukemia is a rapidly progressing disease that impaired cellular functions of mostly the immature blood cells. Chronic leukemia, as implied from its name, progresses slowly as it affects cell types that are better differentiated, thereby more capable in retaining their normal functions (Hoffbrand *et al.*, 2001). Based on their involvement in the hematopoietic cell lineages, acute and chronic leukemia can be subdivided into four distinct groups, namely acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphoid leukemia (CLL) and chronic myeloid leukemia (CML) (Hoffbrand *et al.*, 2001) (Table 1.1).

Figure 1. 3 *Approximate distribution of childhood and adult leukemias.*



(Calculated from "Cancer Facts Figures, 2005, American Cancer Society" [Numbers do not add up to 100 because of rounding.])

Table 1. 1 *Predominant abnormal cell types in four major types of leukemia.*

Types of leukemia	Predominant leukemic cells in BM and blood
Acute lymphoblastic leukemia (ALL)	Lymphoblasts
Acute myeloblastic leukemia (AML)	Myeloblasts
Chronic lymphoid leukemia (CLL)	Lymphocytes
Chronic myeloid leukemia (CML)	Neutrophils, basophils, eosinophils, mekakaryocytes

1.4. Childhood Acute Lymphoblastic Leukemia (ALL)

1.4.1. Epidemiology

Among the four groups of leukemia, ALL is the most prevalent in childhood (Figure 1.3); among the childhood ALL cases, abnormal clones originating from B

progenitor cells represents 80 % -85 % cases while the remaining 15 % - 20 % clones are derived from T progenitor cells (Poplack, 1993). The prevalence of ALL is particularly high in developed countries such as Italy, the United States, Switzerland and Costa Rica (SEER Cancer Statistics Review, 2005). In United States, the annual incidences of ALL are 6.1 per million in American white children and 5.1 per million among the American black children (Chan and Pui, 2003). In Hong Kong, we have a similar incidence at 4 per million children, in other words; roughly 50 to 60 children are diagnosed to suffer from leukemia every year (Children Cancer Foundation, Hong Kong). On the other hand, the occurrence of childhood ALL peaks at two to five years old (SEER Cancer Statistics Review, 2005). In addition, the prognosis for girls with ALL is slightly better than for boy ALL patients (SEER Cancer Statistics Review, 2005); and ALL white children survive better than ALL black children (Chessells, 1995), though the causes are still relatively unknown (Redaelli *et al.,* 2005).

1.4.2. Causes and Risk Factors

As in all types of cancers, both environmental factors (e.g. exposure to carcinogens) and genetic elements (e.g. up-regulation of oncogenesis or down-regulation of tumor suppressor genes) participate in the development of ALL (Balmain *et al.*, 2003). Table 1.2 summarized the current knowledge about the causes

of childhood ALL. However, precise etiology of children ALL is still unknown, as previous studies have only examined risks factors of general ALL, unexploring the specific ALL lineage and subtype. There is little evidence to show a strong association between the environmental factors and the development of ALL, though many investigators have attempted such correlation. Other suggestive factors such as old maternal age, maternal smoking during pregnancy and high birth weight have been reported, but the evidence are neither strong nor conclusive (Sandle and Ross, 1997; Ji *et al.*, 1997; Hjalgrim *et al.*, 2003).

Table 1. 2 *Current knowledge about the risk factors of childhood ALL.*

Factor	Example	Reference
Genetic factors (known risk factors)		
Genetic disease	Down Syndrome patient has 20-fold increase risk	Arceci , 2002
Siblings or identical twins	Siblings or identical twins of affected child have 2 to 4 times greater risk	Sandler and Ross, 1997
Environmental factors (potential risk factors)		
Ionizing Radiation	X-ray exposure during pregnancy	Shu *et al.*, 2002
Chemical	Benzene, trichloroethylene	Schnatter *et al.*, 2005

1.4.3. Molecular Pathophysiology

ALL, resulting from the clonal expansion of genetically and abnormally altered lymphoid progenitor cells (Armstrong and Look, 2005), can be characterized by the

rapid accumulation of primitive leukemic blasts in the bone marrow which subsequently invades many tissues, including liver, spleen, lymph nodes, and central nervous system (CNS) (Spiegel *et al.*, 2004). Severe anemia, thrombocytopenia of marked bleeding and great susceptibility to infection are resulted when the abnormal blasts replace normal bone marrow in bone and normal leukocytes in circulation (Hoffbrand *et al.*, 2000).

The role of oncogenes on the induction and maintenance of malignancy is crucial. It has been found that chromosomal translocations, which itself can cause chromosomal abnormalities and thereby the production of oncogenes, are present in as many as 75 % of ALL cases (Armstrong and Look, 2005). Besides, the use of molecular analysis in identifying chromosomal aberrations has also solidified the notion that chromosomal abnormalities are unique to leukemias. These abnormalities lead to differentiation arrest and aberrant growth of leukemic cells. The identification of chromosomal translocations provides a foundation of the molecular pathogenesis of ALL. The details of chromosomal aberrations found in childhood ALL will be discussed in section 1.4.6.2.

In 1983, the involvement of a tumor suppressor gene in the formation of retinoblastoma is firstly proposed (Benedict *et al.*, 1983), since then, a growing

number of tumor suppressor genes have been discovered as the contributors in tumorigenesis. Tumor suppressor genes are altered via different mechanisms, including deletions and point mutations, which may result in an inactivation of particular protein (Stanbridge, 1990). Epigenetics regulation of the tumor suppressor gene can also blunt its expression (Garinis, 2005). A broad spectrum of tumor suppressor gene alterations do occur in ALL, especially structural alterations of retinoblastoma suppressor gene (*Rb*) gene (Sauerbrey *et al.*, 1998), *p15* (Cayuela *et al.*, 1996), *p14* (Faderl *et al.*, 1999) and *p53* (Sugimoto *et al.*, 1991).

1.4.4. Clinical Presentation

Acute leukemia is defined as the presence of over 30 % of blast cells in the BM at clinical presentation (Kojima *et al.*, 1990). The diagnosis of ALL requires general physical examinations (chest X-ray of thymus and lumbar puncture) and BM assessments (complete blood count analysis, cytochemical and morphological evaluation, immunophenotyping, cytogenetics and molecular genetic analysis) (Gilliland and Tallman, 2002).

The symptoms of ALL are fairly non-specific. The most common features of childhood ALL are lymphadenopathy, hepatomegaly, splenomegaly and fever. Other

clinical features of ALL include anemia and hematostatic dysfunction (Pui, 2001).

1.4.5. Classification

1.4.5.1. Immunophenotyping

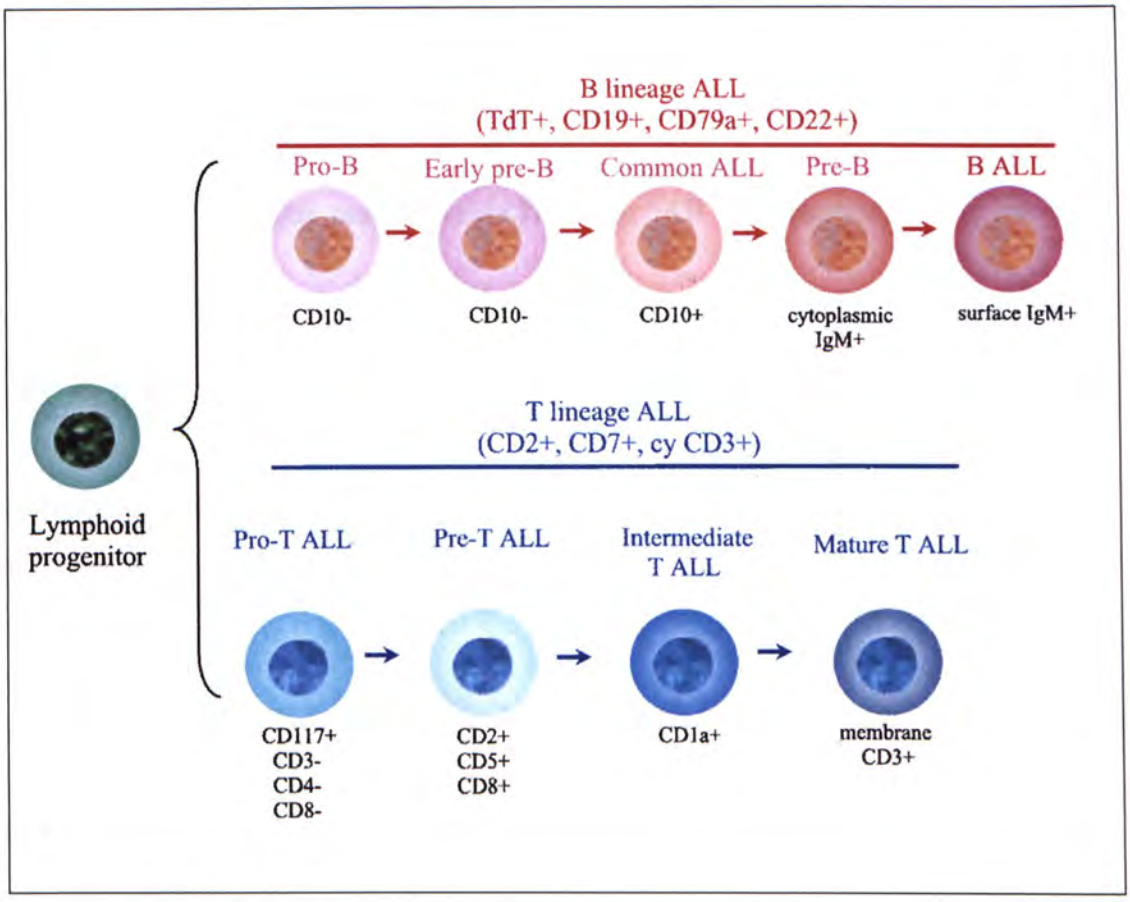
Most leukemic cells share similar features with normal lymphoid precursors, since then, the use of combinatory immunological markers is useful to sub-classify B lineage ALL and T lineage ALL into different subtypes (General Hematology Task Force of the BCSH., 1994; Farahat *et al.*, 1995; Bene *et al.*, 1998). The B lineage ALL is characterized by the expression of at least two of the B-cell antigens CD19, CD79a or cytoplasmic CD22 with lack of expression of T and myeloid cell markers. In addition, B lineage ALL is sub-classified into two main types: precursor B-cell derived ALL and B-ALL (also known as Burkitt's leukemia) (Ludwig *et al.*, 1994). The precursor B-cell ALL is further subtyped by immunophenotyping: pro-B ALL, early precursor-B cell (early pre-B) ALL, common ALL and precursor-B (pre-B) ALL. T lineage ALL, whose shows expression of T-cell antigens like CD7 and cytoplasmic CD3 is sub-classified into four subtypes: pre T-ALL, early T-ALL, intermediate T-ALL and mature T-ALL (Ludwig *et al.*, 1994). The specific markers used to distinguish ALL subtypes are illustrated in Figure 1.4.

Generally, precursor B-cell ALL have a more favorable prognosis than others

B-ALL and T lineage ALL. However, there are still subsets of B-cell precursor ALL patients come with poor prognosis such as those patients having CD10 positive expression (Pui, 1995).

Immunophenotyping can be used to detect morphologically and cytochemically unclassified ALL. In particular immunophenotyping is a major factor in determining the chemotherapy protocol of a patient (Coustan-Smith *et al.*, 2000). Flow cytometry, an important individual cell monitoring technology nowadays, allows the detection of minimal residual disease (MRD) which is particularly important in identifying prognostic groups and distinctive individualized treatment of a ALL patients (Mortuza *et al.*, 2002). The concepts of MRD are defined as malignant cells that cannot be detected by morphological examination but can be identified with newer technology instead (Dworzak *et al.*, 2002). The advantage of using multi-parametric flow cytometry is its capability in detecting 1 leukemic cell among 10000 normal cells, permitting precise immunophenotypic characterization of leukemic cells even the malignant cells are in low numbers (Riley *et al.*, 2002).

Figure 1. 4 *Sub-classification of ALL.*



(Summarized from Ludwig et al., 1994)

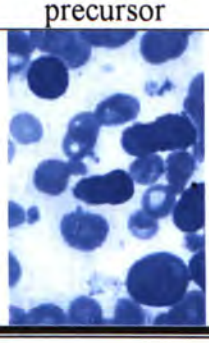
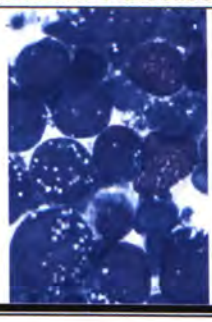
1.4.5.2. French-American-British (FAB) Classification

Childhood ALL patients can be categorized into three major risk groups, namely, standard risk, intermediate risk and high risk group, according to some known prognostic factors such as white blood cell (WBC) count, age, immunophenotype, genetic aberrations and early response to treatment (Friedmann and Weinstein, 2000; yeoh *et al.*, 2002).

The French-American-British (FAB) classification has been introduced for more

than 25 years. It is based on the morphological, cytochemical and immunophenotypic presentation of ALL and AML (Bennett *et al.*, 1976). This classification, together with clinical presentations, is a useful predictor for preliminary diagnosis of ALL and help to establish patient's risk profile (Table 1.3). The FAB classification system has been adopted internationally. Another classification system developed by the World Health Organization (WHO) includes cytogenetics molecular genetics data, as well as morphology and immunophenotyping (Mathe and Rappaport, 1976; Harris *et al.*, 1997). However, the WHO classification has never been widely used due to its debatable utility.

Table 1. 3 *FAB classification of ALL.*

	L1	L2	L3
Frequency	80 %	17 %	3 %
Cell size	Small and homogeneous	Large, often heterogeneous	Large, heterogeneous
Amount of cytoplasm	Scant	Moderately abundant	Moderately abundant
Nucleoli	Small and not prominent	Numerous and prominent	Present and may be prominent
Cytoplasmic vacuoles	Variable	Variable	Prominent
Immunophenotype association	B-cell or T-cell precursor	B-cell or T-cell precursor	Mature B- cell (Burkitt's disease)
Representative microscopic images			

(Summarized from Pui and Crist, 1994; Mckenna, 2000)

1.4.6. Diagnosis and Prognosis

Prognostic features play a critical role in the effectiveness of childhood ALL therapy. It is important to know the risk group of childhood ALL patient in order to plan individualized treatment (Friedmann and Weinstein, 2000). Advances in medicine have improved the prognosis of children ALL from 15 % in the late 1960s to approximately 80 % in some cases today (Pui *et al.*, 2004). Recent findings have shown that the prognosis of pediatric ALL patients are usually better when compared to adult patients (Pui *et al.*, 2004), as only 60 % adult ALL patients can achieve

long-term disease-free survival (DFS) (Redaelli et *al.*, 2005; Hoelzer and Gokbuget, 2000). The poor outcome in adult ALL has been attributed to the increased toxicity of drugs, the lower tolerable doses and shorter cycles of treatment (Thomas *et al.*, 2001). Studies have suggested different disease biology, host features, treatment strategies, early response to treatment, specific subtype and age are the leading reasons for such varied treatment response between the pediatric and adult ALL patients (Downing and Shannon, 2002).

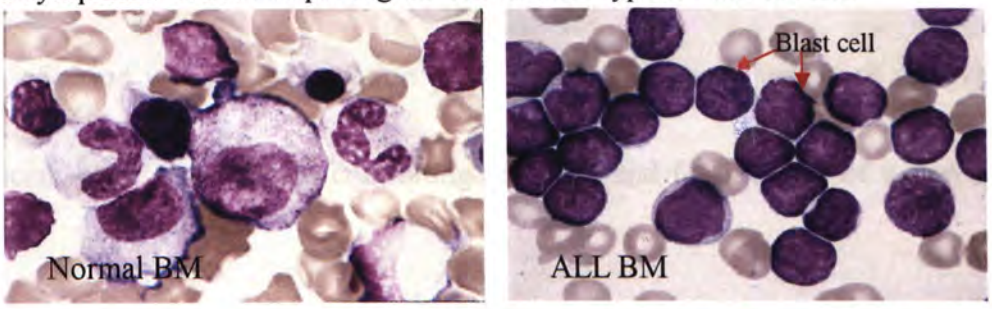
Although the prognostic factors based on age and WBC count act as a powerful predictors of disease outcome, there are still 20 % of standard risk childhood ALL patients destined to relapse by current therapy (Pui and Evans, 1998). Therefore, scientists have moved to investigate the prognostic significances based on better refined immunophenotypes and genetic features. The prognostic impact of these factors will be discussed in this section.

1.4.6.1. Morphological and Cytochemical Analysis

The diagnosis of ALL can usually be achieved by careful morphological assessments of BM aspirate smear (Figure 1.5) (Tosi *et al.*, 1987). Moreover, cytochemical analysis is often used to distinguish poorly differentiated ALL. Table 1.4 shows the morphological and cytochemical profiles of ALL (McKenna, 2000).

Figure 1. 5 *Microscopic examination of normal and ALL BM specimens.*

The blast cells in ALL patients are relatively small, have condensed chromatin and little cytoplasm. These morphological features are typical case of ALL.



(Adapted form the website http://www-sdc.med.nagasaki-u.ac.jp/n50/disaster/Leukemia-E.html)

Table 1. 4 The morphological and cytochemical profiles of ALL patient.

Morphological profile	
Blast size	Small to medium; variable in size
Chromatin	Rather coarse
Nucleoli	Absent or one or two; indistinct
Cytoplasm	Scant or moderate
Auer rods	Absent
Myelodysplasia	Absent
Cytochemical profile	
Myeloperoxidase and Sudan Black B	Negative
Non-specific esterase	Focal; not definitive
Periodic acid Shiff (PAS)	Positive; 75 %
Acid-phosphatase	Positive in T-ALL

(Summarized from Hoffbrand et al., 2001 and McKenna, 2000)

1.4.6.2. Cytogenetic and Molecular Genetic Characterizations

Cytogenetic and molecular techniques has been begun to contribute in the diagnosis, prognosis and classification of ALL (Table 1.5). Cytogenetics techniques that are adapted for current clinical usage include fluorescence in situ hybridization

(FISH), spectral karyotyping (SKY) and comparative genomic hybridization (CGH) (Gilliland and Tallman, 2002). Specifically, a wide variety of translocations in ALL were occurred via: (1) deregulation of an intact gene by disruption or removal, (2) replacement of the adjacent controlling elements and (3) creation of a new fusion gene.

Approximately one-third of ALL blasts shows an increased chromosome numbers (e.g. hyperdiploid, >47 or >50 chromosome). Besides, one-third of the ALL blasts show chromosomal translocations (Carroll *et al.*, 2003). The most commonest rearrangements in childhood ALL are illustrated in Table 1.6. *TEL-AML1* translocation is the most prevalent molecular cytogenetics abnormality in childhood ALL (Downing and Shannon, 2002).

Genetic abnormalities found in the ALL blasts provide an insight into the prognosis of ALL especially the prediction of sensitivity towards chemotherapeutic agents (Raimondi, 1993; Zwaan *et al.*, 2002). For example, in terms of disease biology of precursor B-cell ALL, *TEL-AML1* translocation is the most prevalent genetic abnormalities among pediatric patients and these patients can usually be cured with chemotherapy alone (Krishna *et al.*, 2001). However, in adult precursor B-cell ALL, investigators have observed high percentage of *BCR-ABL* fusion, which is known to possess a strong chemoresistant behavior that can diminish chemotherapy

response in adult patients (Westbrook *et al.*, 1992).

More recent report demonstrates the chromosomal rearrangement across 11q23 is associated with a poorer prognosis and it contributes to 75 % of infant ALL cases (Pui *et al.*, 2002). In particular, the presence of Philadelphia chromosome in Ph ALL patients has the worst prognosis among all childhood leukemias, and recurrence usually occurs in these patients (Arico *et al.*, 2000).

Table 1. 5 *The relationship between cytogenetics and prognosis of ALL.*

Prognosis	Types of chromosomal abnormalities
Standard risk	Hyperdiploidy >50
	TEL-AML1 (t(12;21)(p13;q22))
Intermediate risk	Standard risk in age and WBC without significant genetic risk features
High risk	*E2A-PBX* (t(1;19)(q23;q13))
	T lineage ALL
	High risk in age and WBC without significant genetic risk features
Very high risk	*BCR-ABL* (t(9;22)(q34;q11))
	MLL rearrangement

(Summarized from Armsstrong and Look, 2005; 2000 and McKenna, 2000)

Table 1. 6 *Commonest chromosomal translocations in childhood ALL.*

Chromosomal translocation	Fusion genes	Predominant types of leukemia	Frequency	Reference
t(12;21)(p13;q22)	*TEL-AML1*	Precursor B-cell ALL	22 %	Golub *et al.*, 1995
t(4;11)(q21;q23)	*MLL*	Infant ALL	8 %	Pui *et al.*, 2002
t(1;19)(q23;q13)	*E2A-PBX*	Pre-B ALL	5 %	Uckun *et al.*, 1998
t(9;22)(q34;q11)	*BCR-ABL*	Ph ALL	3 %	Hilden and Kersey, 1994
TAL1 deletion	*SIL-TAL1*	T-ALL	20 %	Hoelzer, 2002

1.4.7. Treatment

Different types of treatments are used for treating childhood ALL. These include chemotherapy, radiation therapy and stem cell transplantation (Pui, 1998). Most chemotherapy treatment protocols include three phases: induction, intensification and maintenance phases (Table 1.7.) (Redaelli *et al.*, 2005). These three phases create a complete remission of 99 % (Pui and Evans, 1998). The CNS is the second most frequent site of leukemia relapse besides BM. Therefore, CNS prophylaxis is given during each phase of therapy to kill leukemic cells in the CNS (Dritschilo *et al.*, 1976).

The latest chemotherapy regimen is adopted from the HKALL97 protocol, developed by the Hong Kong Pediatric Hematology and Oncology Study Group. This protocol is originated from the German Berlin-Frankfurt-Muenster 95 (BFM95) protocol that includes a delayed intensification similar to the induction phase repeated

5 months after diagnosis (Li *et al.*, 2006). This protocol has demonstrated a decreased relapse rate compared to previous protocols (15.7 % vs. 37.3 %; $p<0.001$) (Li *et al.*, 2006).

For instance, 40 % of childhood ALL patients fall into low risk group and they often require lower dose of chemotherapy, less toxic medications and less intensive CNS prophylaxis. However, around 9 % of childhood ALL patients fall into the high risk group and they will be subjected to stem cell transplantation (Rubnitz and Look, 2000).

Table 1. 7 *Three phases of chemotherapy for childhood ALL patient.*

Phases	Description	Medications used
Induction phase	This phase intends to kill leukemic cells in BM and blood.	Steroids (prednisone, prednisolone, dexamethasone)
Intensification phase	It begins after complete remission and is used to kill any remaining inactive leukemic cells to prevent relapse.	Methotrexate, 6-mercaptopurine, L-asparginase, dexamethasone, vincristine, doxorubicin, thioguanine and cytarabine.
Maintenance phase	It is used to kill any residual leukemic cells that would proliferate and cause relapse.	Methotrexate, 6-mercaptopurine
CNS prophylaxis	Includes intrathecal chemotherapy and radiation	Methotrexate and cytarabine

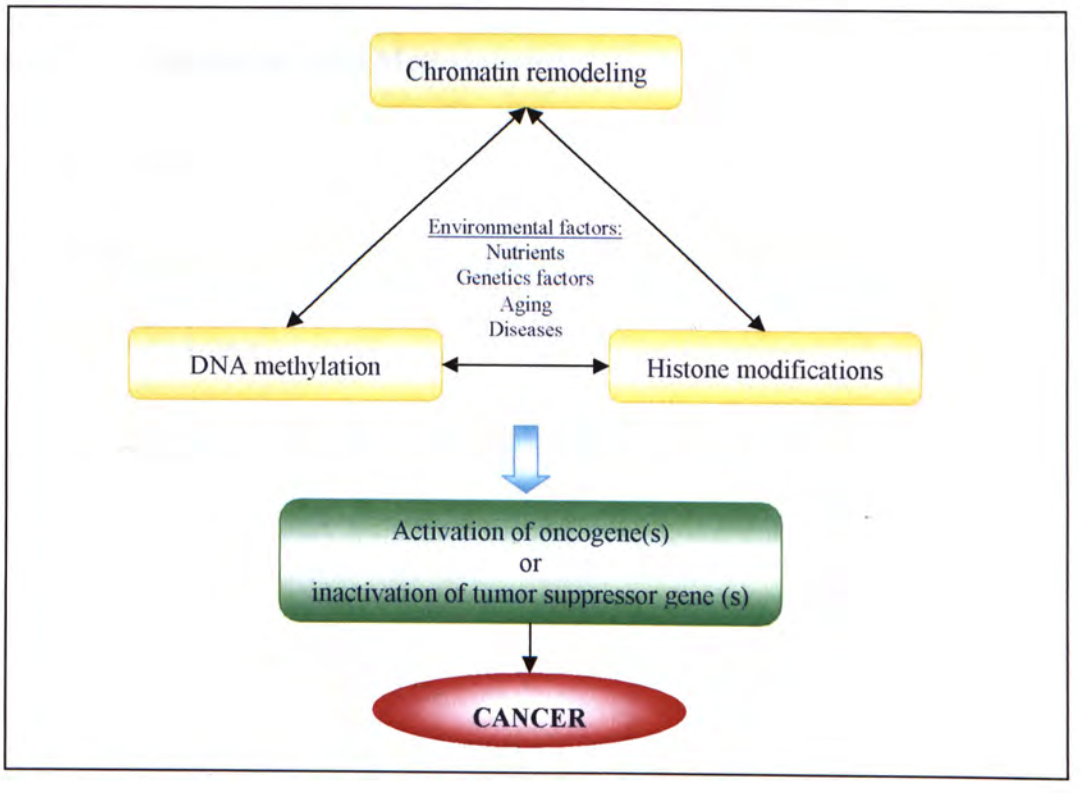
(Summarized from Redaelli et al., 2005)

1.5. Overview of Epigenetics

The principle and mechanism of Epigenetics was firstly introduced in 1983 (Feinberg and Vogelstein, 1983; Gama-Sosa, 1983). Epigenetic inheritance is defined as cellular information, other than the DNA sequence itself that is heritable during cell division, in other words, is capable to alter the gene expression without a change in nucleotide sequence (Feinberg and Tycko, 2004).

In the succeeding years, the concept of epigenetic has become more prevalent when researchers have further demonstrated the participation of epigenetic events in many physiological and pathophysiological conditions, as implied from human cancers (Reik *et al.*, 2001; Laird, 2005). Epigenetics involves three mechanisms: DNA methylation, post-translational modification of histone (methylation, phosphorylation and acetylation) and chromatin alteration (modification of protein binding molecules) (Gius, 2005). These mechanisms may take part in tumorigenesis either through the inactivation of tumor suppressor genes or the activation of oncogenes (Osada and Takahashi, 2002; Esteller, 2006) (Figure 1.6).

Figure 1. 6 *Epigenetic regulation of gene expression in tumorigenesis.*



1.6. Concepts of DNA Methylation

Cytosine DNA methylation is the commonest epigenetic events in the mammalian genome. DNA methylation, occurring primarily at the cytosine residue at 5'-CG-3' nucleotides, has important roles in the regulation of gene expression, genomic imprinting, as well as silencing of repeating elements in the genome.

1.6.1. CpG Islands

CG site is a dinucleotide sequence of 5'-CG-3' that can be found throughout the vertebrate genome, though presents at a relatively low frequency. It has been reported that the actual presence of 5'-CG-3' dinucleotide sequences occurred within 5% to 10 % of its predicted frequency due to the high mutability of methylated cytosine (Das and Singal, 2004).

CpG islands, a cluster of CG sites firstly described in 1987 by Gardiner-Garden and Frommer, is a region longer than 200bp with GC content greater or equal to 50 % and a CpG observed/expected ratio greater or equal to 0.6 (Gardiner-Garden and Frommer, 1987). It can be found occasionally throughout the human genome. A CpG island is usually kept unmethylated with the exception of inactive chromosome X in females. In mammal, approximately 60 % of the gene promoters are co-localized with

a CpG island which suggests its importance in the regulation of a particular gene (Antequera, 2003).

1.6.2 Mechanisms of DNA Methylation

DNA methylation is a covalent modification of nucleotides, in which a methyl group is transferred from *S*-adenosylmethionine (SAM) to the carbon-5 position of cytosine by DNA-methyltransferases (DNMTs) (Vilkaitis *et al.*, 2001) (Figure 1.7). The underlying mechanism of DNA methylation is still controversial. There are three proposed mechanisms to account for the transcriptional repression by DNA methylation.

The first mechanism involves a direct interaction between specific transcriptional factors (AP-2, c-Myc/Myn, cyclic AMP-dependent activator CREB, E2F and NFκB) with their DNA recognition sites at the corresponding promoter sequence. These transcriptional factors have a recognition site that recognizes 5'-CG-3' dinucleotide. It has been shown that methylation can inhibit the interaction between transcriptional factors and the promoter sequences (Figure 1.8-A) (Tate and Bird, 1993).

The second possible mechanism is the involvement of direct binding of specific transcriptional repressors to methylated DNA sequence. This interaction is mediated by methyl cytosine binding proteins (MeCP), which targets the 5' methylated CpG

sequence (Prokhortchouk and Hendrich, 2002). These proteins include MeCP1, MeCP2, methyl-CpG binding domain protein (MBD) 1, MBD2, MBD4 and Kaiso (Figure 1.8-B) (Das and Singal, 2004).

The third mechanism suggests that DNA methylation can mediate histone modification and chromatin structure and hence altering gene expression. The acetylation status of the histone plays an important role in the transcriptional regulation (Fraga and Esteller, 2005). Being the major component of chromatin, histone can be acetylated or deacetylated by histoneacetylases (HATs), the gene activator, or histonedeacetylases, the gene repressor (HDACs), respectively (Kouzarides, 2002; Grunstein, 1997). MBD proteins and DNMT1 recruits HDACs to the methylated promoter. Also, methylation of histone-H3-lysine 9 (H3-K9) by histone methylases is associated with chromatin condensation and suppression of gene expression (Nakayama *et al.*, 2001). This model suggests that DNA methylation not only prevents the activation of gene by blocking the access of transcriptional factors but also stabilizing the transcriptionally inactive state of chromatin (Singal and Ginder, 1999) (Figure 1.9).

However, it is now realized that hypermethylation at CpG island is just one of the consequences of an integrated change in chromatin structure and histone modifications (Lund and van Lohuizen, 2004). Nevertheless, the understanding of the

status of DNA methylation is a useful marker for assessing the epigenetic state of a locus, because it is preserved in DNA isolated from cell, and can be measured by PCR-based techniques easily.

Figure 1. 7 *Conversion of cytosine to 5'-methyl-cytosine by DNMT.*

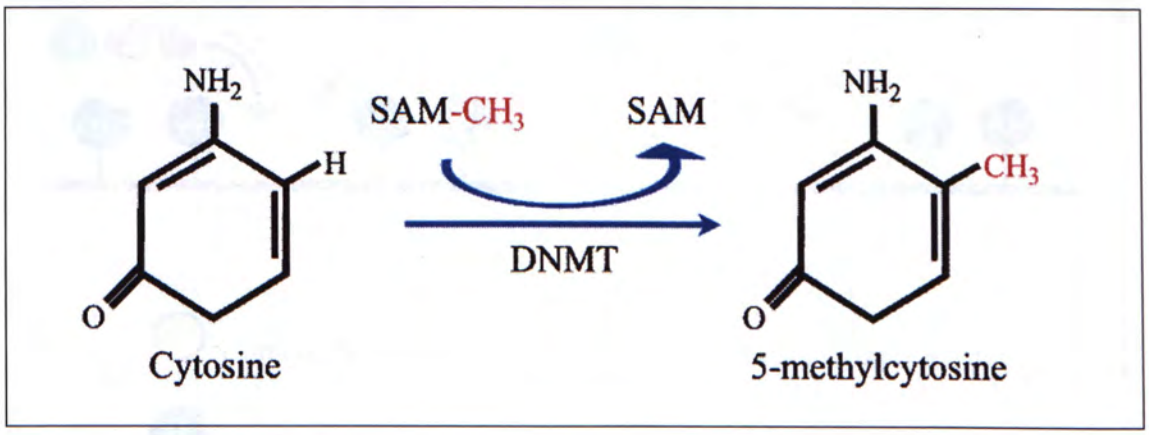


Figure 1.8 *The two proposed DNA methylation mechanisms regulating the transcriptional repression by transcription activators and repressors.*

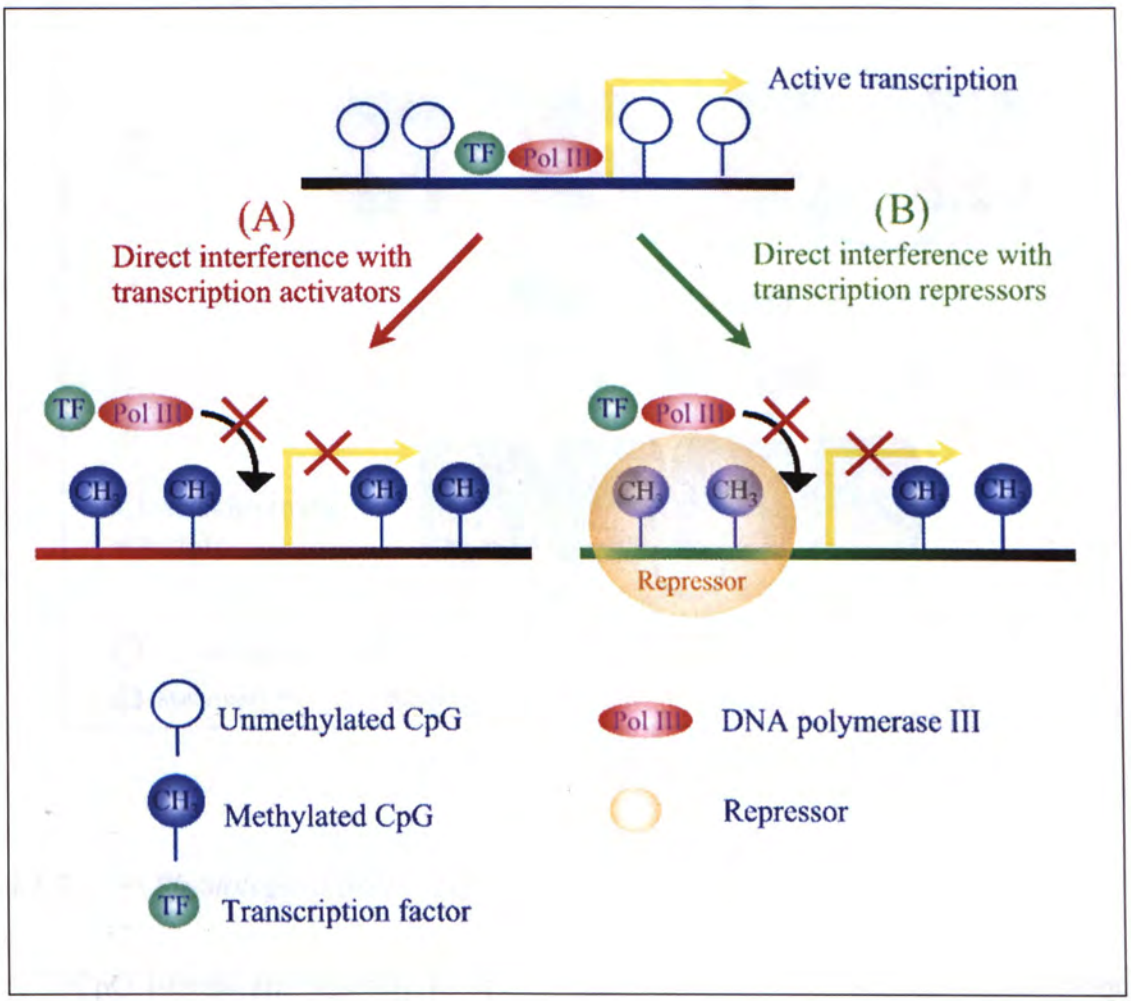
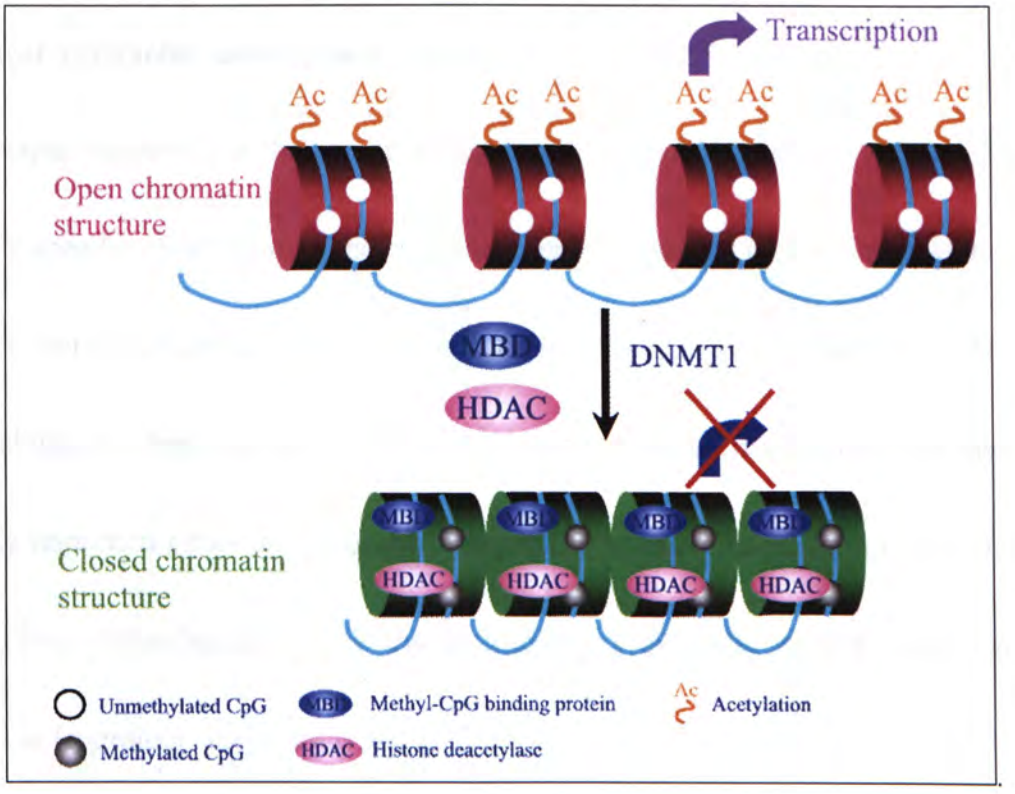


Figure 1. 9 *Chromatin modulation mediated by DNA methylation.*



1.6.3 Physiological Roles of DNA Methylation

CpG islands are typically located at the 5' end of genes and are usually kept unmethylated. In contrast, the majority of methylated CpG islands are located within the repetitive elements including centromeric repeats, satellite sequences and gene repeats that encode ribosomal RNAs (Holmes and Soloway, 2006). Genomic imprinting and X-chromosome inactivation are the most prominent examples of methylation-regulating transcriptional repression in normal cells (Lyon, 1994). In this section, the roles of DNA methylation in normal physiological development will be discussed.

The proper control of DNA methylation is responsible for genomic imprinting in normal embryonic development (Okano *et al.*, 1999; Swales and Spears, 2005). Genomic imprinting is the parent-of-origin specific gene expression, whose controls allele-specific expression of the imprinted genes by means of DNA methylation. In other words, either the paternal or maternal allele is active for normal embryonic development. Approximately 80 imprinted genes have been identified and many of these imprinted genes are involved in regulating resource acquisition of the embryo and fetus (Horsthemke and Ludwig, 2005). For example, *human small nuclear ribonucleoprotein polypeptide N* (*SNRPN*) is highly methylated in oocytes but unmethylated in sperm. Specifically, hypermethylation of *SNRPN* was found to be proportional to oocyte diameter (Lucifero *et al.*, 2004).

X-chromosome inactivation, on the other hand, is another important biological significance of DNA methylation. The concept of X-chromosome inactivation is to inactivate one of the two female X-chromosomes so as to maintain chromosomal balance. Precisely, it ensures equivalent gene expression level between female and male. This stable inactivation has proven to be resistant to drug treatment and selection (Grant and Chapman, 1988).

The degree of tissue-specific expression is also determined by DNA methylation. Maspin (*SERPINB5*), is a gene generally silenced in certain cell types including skin

fibroblasts, lymphocytes, bone marrow, heart or kidney. On contrary, it does express in epithelial cells, such as in airway, breast, skin, prostate and mouth. This silencing has shown to be unrelated to mutation or deletion. Therefore, hypermethylation of *SERPINB5* turns out to be the cause of regulating tissue-specific expression (Futscher *et al.*, 2002).

DNA methylation at gene deficient regions, such as pericentromeric heterochromatin, plays a crucial role in maintaining the conformation and integrity of the chromosome (Ehrlich, 2000). Moreover, DNA methylation has also been proposed as a genomic defense system against mobile genetic elements like transposons and endogenous retroviruses. It appears as defense against the parasitic sequence elements, which constitutes more than 35 % of the human genome (Bestor, 1998).

1.6.4 Initiation of Aberrant DNA Methylation

The over-expression of DNMTs likely contributes to the aberrant *de novo* methylation and also cancer development. The faulty repair mechanisms mediated by DNMTs may also introduce repairing errors and generate abnormal methylation patterns (Ramchandani, 1999).

DNA methylation is mediated by three DNMT: DNMT1, DNMT3a and DNMT3b (Jones and Baylin, 2002). Aberrant DNA methylation may be caused by the

infidelity of DNMT1, and DNMT1 has been shown to have higher affinity for hemimethylated DNA. It is believed that DNMT1 is the primary enzyme responsible for copying methylation patterns from the parental to the daughter strand in DNA replication (Yokochi and Robertson, 2000). During DNA replication, methylation errors may be introduced and accumulated, causing hypermethylation. In particular, aging is a situation of this kind of progressive increase in the number of methylated CG dinucleotides (Issa, 2000). On the other hand, DNMT3a and DNMT3b have an equal affinity for unmethylated and hemimethylated DNA. However, they may target at the CpG sequence incorrectly and hence contribute to aberrant DNA methylation (Xie *et al.*, 1999).

1.7. DNA Methylation in Tumorigenesis

The roles of DNA methylation had been extensively studied because of its contribution in tumorigenesis (Esteller, 2002). The malignant cells show great discrepancies in DNA methylation status with normal cell. It is found that malignant cells have acquired global hypomethylation and regional hypermethylation was found in specific tumor suppressor genes (Figure 1.10). Aberrant DNA methylation have been observed in several scenarios particularly in cancers, such as colon cancer, lung cancer, leukemia and ovarian cancer (Issa *et al.*, 1993; Belinsky *et al.*, 1996; Melki *et*

al., 1998; Ahluwalia *et al.*, 2001). Numerous reports have demonstrated the consequences of promoter hypermethylation in suppressing or inhibiting gene expression (Esteller, 2002) (Table 1.8). Evidences have shown that the degree of methylation is inversely proportional to the expression of a particular gene (Singal and Ginder, 1999). Besides, hypomethylation also occurs in various subsets of cancers (Hoffmann and Schulz, 2005).

Figure 1. 10 *Hypermethylation and hypomethylation.*

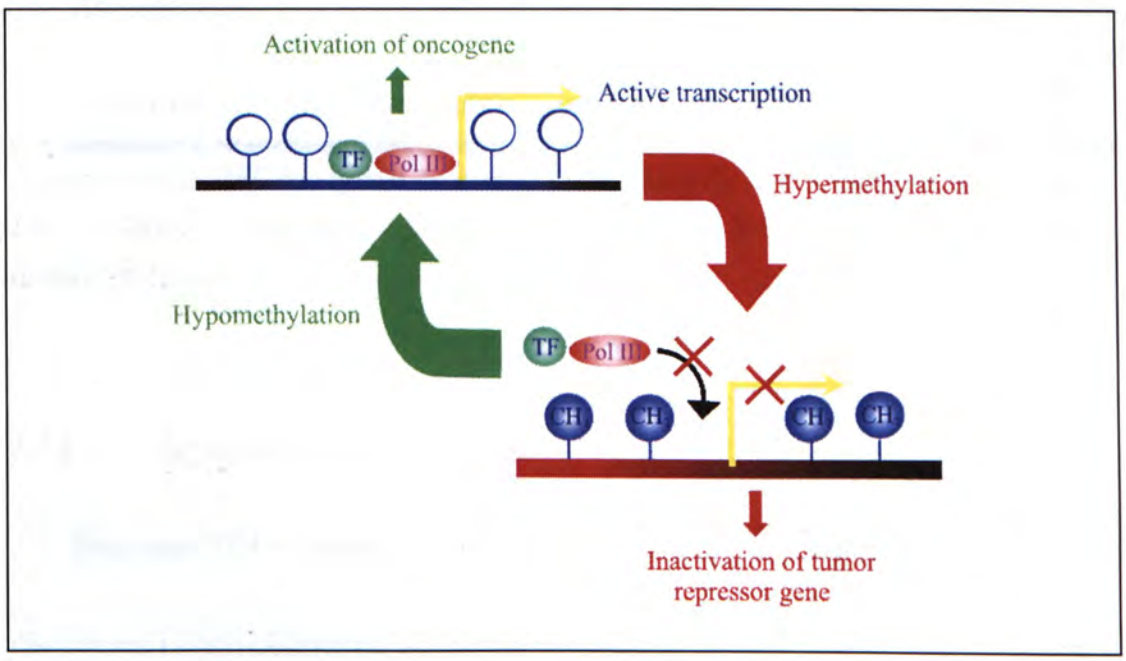


Table 1. 8 *The roles of genes commonly methylated in human cancers.*

Role of cancer development	Gene	Cancers involved	Reference
Cell cycle regulation	*CDKN2A/p16*	Gastrointestinal tract; Head and neck	Herman *et al.*, 1995; Sanchez-Cespedes *et al.*, 2000
DNA repair	*BRCA1*	Breast; ovarian	Dobrovic and Simpfendorfer , 1997; Chan *et al.*, 2002
Drug resistance	*ER*	Breast; prostate	Yang *et al.*, 2001; Li *et al.*, 2000
Detoxification	*GSTP1*	Prostate; renal	Esteller *et al.*, 1998
Differentiation	*p15*	Leukemia; lymphoma	Melki *et al.*, 2000; Herman *et al.*, 1996
Angiogenesis	*TIMP3*	Breast;	Lui *et al.*, 2005; van der Velden *et al.*, 2003
Metastasis	*E-cadherin*	Throid; gastric	Graff *et al.*, 1998; Waki *et al.*, 2002

Abbreviations: *BRCA1,* breast cancer 1; *CDKN2A/p16,* cyclin-dependent kinase 2A; *ER*, estrogen receptor; *GSTP1*, glutathione S-transferase P1; *TIMP3*, metalloproteinase-3

1.7.1. Regional Hypermethylation

Regional DNA hypermethylation of a tumor suppressor gene was firstly discovered in retinoblastoma where the methylation status of the *Rb* was investigated. It was found that hypermethylation of the *Rb* gene was closely linked to the development of retinoblastoma (Greger *et al.*, 1989).

Methylation-regulating genes are those genes involved in cell cycle regulation, DNA repair, drug resistance, detoxification, differentiation, angiogenesis and

metastasis (Baylin and Ohm, 2006). Findings from several studies have shown that tumor cells can maintain the genetically defected allele while the other allele will be potentially inactivated by secondary causes. According to the Kundson's Two-hit Model, bi-allelic inactivation is required for the complete inactivation of a tumor suppressor gene (Knudson, 1996). The genetic inactivation of one allele may be due to the loss of heterozygosity (LOH), deletion, mutation or DNA methylation (Payne and Kemp, 2005). In addition to point mutation and deletion, promoter hypermethylation, on the other hand, is suggested to have an alternative role for transcriptional repression. Upon this circumstance, the remaining alleles will be inactivated, resulting in complete loss of a tumor suppressor gene and finally leading to the development of cancer.

1.7.2 Global and Regional Hypomethylation

Global hypomethylation has been shown to increase with the grade of malignancy (Feinberg and Vogelstein, 1983), whose has been found in cancers such as breast (Soares *et al.*, 1999 ; Szyf *et al.*, 2004), cervical dysplasia and carcinoma (Kim *et al.*, 1994).

Regional hypomethylation is the second most important DNA methylation defect which is observed in various malignancies and solid tumors, including hepatocellular

carcinoma (Saito *et al.*, 2001), cervical cancer (Kim *et al.*, 1994), prostate tumor (Tokizane *et al.*, 2005) and leukemias (Worton *et al.*, 1991). Hypomethylation can contribute to oncogenesis by: (1) activation of oncogenes like *cMYC* and *HRAS*, (2) activation of retrotransponson and (3) chromosomal instability (Cheah *et al.*, 1984; Browett and Norton, 1989) (Figure 1.10).

1.7.3 Microatellite Instability and Oncogeneic Mutation

Besides global hypomethylation and regional hypermethylation, DNA methylation also contributes to microsatellite instability and mutational events during tumor progression (Figure 1.11 and 1.12). This phenomenon was first described in colorectal cancer relating mismatch-repair gene *mutL homolog 1* (*MLH1*) (Kane *et al.*, 1997). The hypermethylation of *MLH1* promoter was found in colorectal cancer patients who have increased microsatellite instability when compared with normal individuals (Kane *et al.*, 1997). It is suggested that hypermethylation does play a role in oncogenic mutation (Magewu and Jones, 1994; Greenblatt *et al.*, 1994). For instance, *O^6-methylguanine DNA methyltransferase* (*MGMT*), is a DNA-repair gene which is silenced by mutational events with combination of the effect from DNA hypermethylation in colon cancer. Hypermethylation of the *MGMT* promoter leads to increased G→A mutation (Esteller *et al.*, 2000). Moreover, hypermethylation occuring

at the coding region of genes also increases the mutational rate (C→T transition, CC→TT mutation and G→T transversion mutation) due to spontaneous deamination, enhanced UV absorption and carcinogen binding near the methylated CpG (Jones and Baylin, 2002). An example is the hypermethylation of *p53* resulted in C→T transition (Greenblatt *et al.*, 1994) (Figure 1.12). Mutation of *p53* was found in 50 % of human cancers and 24 % of these mutations are due to hypermethylation induced C→T transition (Magewu and Jones, 1994).

Figure 1. 11 *Hypermethylation induced chromosomal instability.*

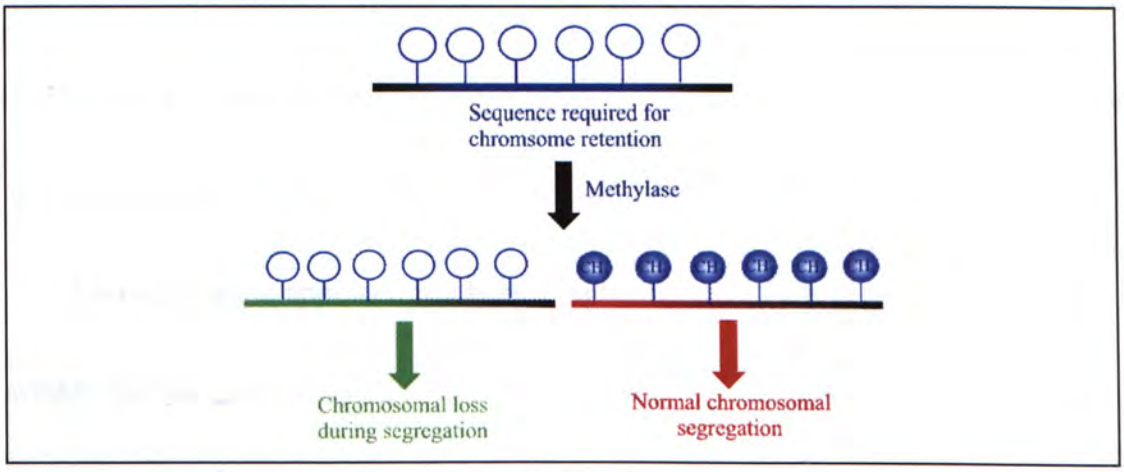
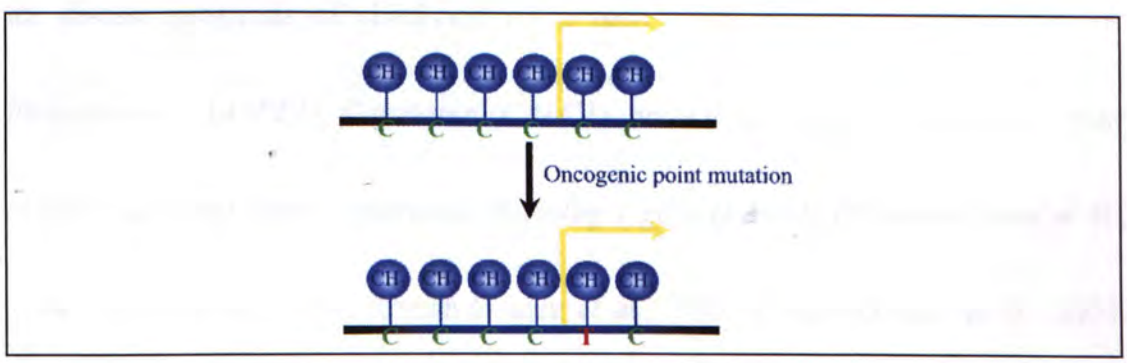


Figure 1. 12 *Hypermethylation induced oncogenic mutation.*



Chapter 2 Literature Review

2.1. Aberrant DNA Methylation in Childhood ALL

Among different types of epigenetic mechanisms, DNA methylation has been studied extensively in the last few years as implied from the transcriptional silencing of tumor suppressor genes in various cancers (Baylin, 2005; Toyota and Issa, 2005; Galm *et al.*, 2006).

It has been found that DNA methylation does play an important role in acute lymphoblastic leukemia (ALL) tumorigenesis (Ponder, 2001; Pui *et al.*, 1990). Therefore, the identification of aberrantly methylated genes is valuable for our understanding in childhood ALL pathogenesis.

Candidate-gene approach has been adopted by most current methylation studies, which include investigation of a single gene or multiple genes concurrently (Roman-Gomez *et al.*, 2004; Canalli *et al.*, 2005; Yang *et al.*, 2006). In addition, several studies have demonstrated the correction of aberrant gene methylation with the disease prognosis of childhood ALL, such genes are known as *p21*, *protein phosphatase 1* (*ASPP1*), *Calcitonin* (*CALC1*), *normal epithelial cell-specific 1 gene* (*NES1*) and *large tumor suppressor, homolog 1 gene* (*LATS1*) (Roman-Gomez *et al.*, 2002; Agirre *et al.*, 2006; Roman-Gomez *et al.*, 2002; Roman-Gomez *et al.*, 2004;

Jimenez-Velasco *et al.*, 2004). Specifically, the multiple-genes approach is aimed to correlate the number of methylated genes with the risk prediction of ALL. Multivariate analysis investigating methylation profile of 15 target genes revealed shorter disease free survival (DFS) ($p<0.0001$) and overall survival (OS) ($p=0.0004$) in patients with ≥4 methylated genes (Roman-Gomez *et al.*, 2004). These results solidified the notion that the differences in DNA methylation of specific molecular pathways may contribute to the prognostic differences occurred in pediatric patients with ALL (Canalli *et al.*, 2005).

For instance, genes that are silenced by promoter hypermethylation may be involved in the inactivation of various biological pathways. Examples include: (1) down-regulation of cell cycle regulating molecules (Drexler, 1998), (2) inhibition of the apoptosis (Krug *et al.*, 2002), (3) alteration of cell-cell adhesion (Roman-Gomez, *et al.* 2003) and (4) dysregulated expression of transcription factors, metabolic enzymes and other putative tumor suppressors (Roman-Gomez *et al.*, 2003).

In order to understand the importance of promoter hypermethylation in childhood ALL leukemogenesis, a comprehensive review on the functional aspects of methylation-regulated genes in childhood ALL will be discussed in this sections (summarized in Table 2.1).

2.1.1. Cell Cycle

Recent advances in cancer biology have clearly demonstrated the development of malignancies as well as their progression, are tightly linked to the alteration of molecular mechanisms in cell cycle (Park and Lee, 2003; Macaluso *et al.*, 2006). Cell cycle represents a series of tightly integrated events regulating cell growth, cell proliferation and DNA repair. The alteration of cell cycle, and hence, the replication process is essential for the cancer progression. Cancers including solid tumors and hematological malignancies have been found to be caused by the dysregulation of cell cycle regulating molecules. In particular, cell cycle is positively regulated by cyclins and negatively regulated by cyclin-dependent kinase (CDK) inhibitors (Schwartz and Shah, 2005).

p15 and *p16* encode the CDK inhibitors which is capable to inhibit G1 phase progression. They compete with cyclin D for binding with CDK4/CDK6, in other words, inhibiting kinase activity of CDK4/CDK6 complex, resulting in dephosphorylation of Rb protein and G1 growth arrest (Furukawa, 2002). Promoter hypermethylation of *p15* has been found to range from 10 % to 67 % of childhood ALL patients by various studies (Wong *et al.*, 2000; Roman-Gomez *et al.*, 2004; Canalli *et al.*, 2005; Yang *et al.*, 2006). Furthermore, OS was found to be diminished in *p15* hypermethylated adult ALL patients (Wong *et al.*, 2000). In addition, the

occurrence of *p15* promoter hypermethylation in T lineage ALL is much more frequent in B lineage ALL (Tsellou *et al.*, 2005; Iravani *et al.*, 1997). On the other hand, variable frequencies of *p16* promoter hypermethylation was found, which ranged from 0 % to 40 % of childhood ALL cases (Wong *et al.*, 2000; Yang *et al.*, 2006).

The downstream effector of *p73* and *p53*, called *p21*, is another CDK inhibitor demonstrated promoter hypermethylation in 39 % (27 out of 68) of childhood ALL patients, since then, hypermethylation of *p21* was conferred to a decrease of mRNA expression in patient samples. Multivariate analysis of potential prognostic factors revealed *p21* methylation status as an independent prognostic factor in predicting DFS of childhood ALL patients (p=0.0001) (Roman-Gomez *et al.*, 2002).

p73 is involved in the regulation of cell cycle, cell death, cell development, carcinogenesis and the sensitivity towards chemotherapy (Pluta *et al.*, 2006). A multiple-genes methylation study on *p73*, *p15* and *p57* revealed the relationship between *p73* and poor prognosis in adult ALL. Methylation study conferred 18 % to 20 % of childhood ALL patients with *p73* promoter hypermethylation (Canalli *et al.*, 2005; Roman-Gomez *et al.*, 2004).

2.1.2. Apoptosis

The ability of a cancer cell to increase its number is not only influenced by cell proliferation but also cell attrition. Apoptosis represents a major cause of this attrition, in other words, the inhibition of this programmed cell death may drive cancer development (Reed and Pellecchia, 2005). The regulation of apoptosis is fundamental to hematopoietic homeostasis because stem cell renewal is continuously counterbalanced by apoptosis (Vaux and Korsmeyer, 1999). Therefore, aberrant regulation of apoptosis has been associated with the development of hematological malignancies (McKenna and Cotter, 1997; Rinkenberger and Korsmeyer, 1997; Hetts, 1998; Hanahan and Weinberg, 2000).

A protein of the ASPP family, ASPP1, is responsible for encoding a protein with apoptotic function. Studies have shown the capability of *ASPP1* in increasing the apoptotic effect of *p53* by the inducing transcription of pro-apoptotic genes (Trigiante and Lu, 2006). Recent report demonstrated the promoter hypermethylation of *ASSP1* in 17 % of childhood ALL patients which was associated with decreased mRNA expression in patients samples, also showed a reduction in DFS (p=0.006) (Agirre *et al.*, 2006).

Death-associated protein kinase 1 gene (*DAPK*) encodes a novel serine/threonine kinase which is required for gamma interferon-induced apoptosis

(Katzenellenbogen *et al.*, 1999). *TMS1* belongs to a growing family of apoptotic signaling molecules, whose cleavage and activation is regulated by its own domain (Conway *et al.*, 2000). *DAPK* and *TMS1* promoter hypermethylation were detected in 10 % of childhood ALL patients (Roman-Gomez *et al.*, 2004; Gutierrez *et al.*, 2003). In addition, the hypermethylation of *DAPK* was statistically lower in T lineage ALL (13 %) than in B-ALL (35 %) (Gutierrez *et al.*, 2003).

Apoptotic peptidase activating factor (Apaf-1) plays an important role in the induction of mitochondrial apoptotic pathway. It forms the central element of the multimeric apoptosome with procaspase 9 and cytochrome c. Deficiency in Apaf-1 protein confers resistance to cytochrome-2, c-dependent apoptosis which is found in a variety of tumors and leukemias (Perkins *et al.*, 1998). For instance, *Apaf-1* promoter hypermethylation has been demonstrated in acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and also ALL, suggesting it is not specific to the types of leukamias (Fu *et al.*, 2003). Aberrant hypermethylation *Apaf-1* of was detected in 35 % of childhood ALL cases (Roman-Gomez *et al.*, 2004).

2.1.3. Tissue Invasion and Metastasis

Cell-cell interaction is generally reduced in human cancers. The reduction of intercellular interaction allows cancer cells to disobey their original order, resulting in

destruction of histological structure, which is a morphological hallmark of malignancies (Hirohashi and Kanai *et al.*, 2003). Reduced intercellular interaction is also indispensable for cancer invasion and metastasis. The capacity of invasion and metastasis in solid tumors enables cancer cells to escape the primary tumor mass, invades adjacent tissues and travels to distant sites where nutrient and space are not limiting. This migratory capability causes 90 % of human cancer death (Hirohashi and Kanai, 2003).

Promoter hypermethylation of two cadherin family members genes, *E-cadherin* and *H-cadherin*, have been observed in various cancers as well as in childhood ALL (Kim *et al.*, 2005). The occurrence of aberrant methylation for the two genes were both 32 %, since then, a strong correlation of the methylation status between two genes was confirmed (Roman-Gomez *et al.*, 2004).

Estrogen is known as a negative regulator of normal hematopoiesis. *Estrogen receptor gene* (*ER*) is frequently altered in human hematopoietic malignancies (Issa *et al.*, 1997). It has been shown that *ER* has growth and metastatic suppressor activity in many different cell types including breast, ovarian, prostate endometrial and colorectal cancer (Deroo and Korach, 2006). Also, it is aberrantly hypermethylated in 86 % of human hematopoietic malignancies, including 8 out of 9 childhood ALL cases (Issa *et al.*, 1996).

2.1.4. Transcription Factors and Metabolic Enzymes

Interleukin-12 (IL-12) is an important cytokine that is important in the regulation of innate resistance and adaptive immunity (Trinchieri, 2003). This cytokine is produced by antigen-presenting cells, T-cells, NK cells and B-cells. Silencing of the *Interleukin-12 receptor 2 gene (IL-12R2)* provides growth advantage to malignant cells, where this is a consistent feature in pro-B, early pre-B, and pre-B childhood ALL. Loss of *IL-12R2* mRNA expression has been shown to associate with promoter hypermethylation, which occur in 100 % of childhood B-lineage ALL (Airoldi I *et al.*, 2006).

CALC1 is a metabolic enzyme and is generally not considered as a factor of tumorigenesis (Leegwater *et al.*, 1997). On contrary, *CALC1* hypermethylation was conferred in 39 % (25 out of 49) childhood ALL patients and multivariate statistical analysis confirmed hypermethylation of *CALC1* was associated with shorter DFS ($p<$ 0.00001) (Roman-Gomez J *et al.*, 2003).

2.1.5. Putative Tumor Suppressor Genes

Phosphatase and tensin homolog gene (*PTEN*) is an important tumor suppressor which is contributes to cell adhesion, apoptosis, angiogenesis, G1 cell cycle regulation and signal transduction in the pathogenesis of a number of familial and sporadic

cancers (Zysman, 2003). Study has demonstrated the occurrence of *PTEN* promoter hypermethylation in 20 % of childhood ALL cases (Roman-Gomez J *et al.*, 2004).

NES1 is a putative tumor suppressor gene located in chromosomal region 19q13.3-4. The *NES1* gene was identified by virtue of its down-regulation in breast cancer cell lines, and is considered to be involved in the regulation of normal cell growth (Roman-Gomez J *et al.*, 2004). However, the precise physiological function of NES1 protein is still unclear. Methylation of the *NES1* has been found in 39 % of childhood ALL cases. Moreover, down-regulation of mRNA expression has also been observed. Patients with methylation of the *NES1* had a poorer DFS than unmethylated patients as implied from multivariate analysis revealing shorter DFS (p=0.005) in those patients (Roman-Gomez J *et al.*, 2004).

The human homolog of the drosophila warts tumor suppressor gene, *LATS1*, is responsible to encode a novel serine/threonine kinase and functions as a component of the mitotic apparatus (Bothos *et al.*, 2005). *LATS1* promoter hypermethylation was observed in 30 % of childhood ALL patients. This aberrant methylation was associated with decreased mRNA transcript in patient samples. Also, a decrease of DFS (p=0.0005) and OS (p=0.0003) were found in pediatric patients with ALL having *LATS1* promoter hypermethylation (Jimenez-Velasco *et al.*, 2004).

Dickkopfs-3 gene (*Dkk-3*) is a newly characterized mortalization-related gene

and an antagonist of the Wnt oncogenic signaling pathway, whose expression is decreased in a variety of cancer cell lines suggesting its role in tumorigenesis (Roman-Gomez J *et al.*, 2003). *Dkk-3* methylation was detected in 32 % of childhood ALL cases by a later study, in which, however, decrease of *Dkk-3* mRNA expression was observed simultaneously (Roman-Gomez J *et al.*, 2004).

Parkin is associated with autosomal recessive juvenile Parkinsonism. Functional analysis has shown the contribution of parkin activity in E3 ubiquitin ligase (Cesari R *et al.*, 2003). The promoter hypermethylation of *Parkin* was found to represent 30 % of the childhood ALL cases (Roman-Gomez J *et al.*, 2004).

2.1.6. Chromosome Instability

A growing number of genes involved in sensing and repairing DNA damage, or genes involved in assuring the correct chromosomal segregation during mitosis, have been found to be inactivated in different cancers (Lengauer *et al.*, 1998). Their loss of function causes genome instability, leading to the generation of tumor cells.

The *human fragile histidine triad gene* (*FHIT*) is a putative tumor suppressor gene located at chromosome region 3p14.2. It spans the most inducible human common fragile site, FRA3B (Stam *et al.*, 2006). FHIT protein was conferred to

induce apoptosis and retard tumor cell proliferation in both *in vitro* and *in vivo* studies (Dumon *et al.*, 2005). Among leukemias, hypermethylation of *FHIT* was strongly correlated with ALL histology ($p = 0.008$), high hyperdiploid ($p < 0.0001$) and translocation-negative ($p < 0.0001$) categories (Zheng *et al.*, 2003). Besides, hypermethylation of *FHIT* was found in 27.4 % (52 out of 190) of BM samples from childhood leukemias patients. *FHIT* mRNA and protein expression were found to be down-regulated in patients samples synchronously. Specifically, methylation study on childhood ALL cases revealed *FHIT* promoter hypermethylation was observed in 32 % of childhood ALL cases (Zheng *et al.*, 2003).

Human neuronatin gene (*NNAT*) was mapped to chromosome region 20q11.2-q12 and this region often exhibits loss of heterozygosity in hematological malignancies (MacGrogan *et al.*, 2001). The study on the methylation status of *NNAT* gene in childhood ALL showed 69 % of promoter hypermethylation and aberrant methylation was associated with the decrease of mRNA transcript in patient samples (Kuerbitz *et al.*, 2002).

Table 2. 1 *Details summary about the methylation-regulated genes found in childhood ALL.*

Gene	Methylation frequency	Hypermethylation in correlation with expression in clinical samples	Correlation with clinical parameter in childhood ALL	References
Cell Cycle				
p14	5 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
p15	67 %	Not mentioned	Decreased OS in adult ALL	Wong *et al.*, 2000
	20 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
	15 %	Not mentioned	Not mentioned	Canalli *et al.*, 2005
	10 %	Not mentioned	Not mentioned	Yang *et al.*, 2006
p16	0 %	Not mentioned	Not mentioned	Wong *et al.*, 2000
	40 %	Not mentioned	Not mentioned	Yang *et al.*, 2006
p21	39 %	Decreased mRNA expression	Reduced DFS (p=0.0001; for both adult and childhood)	Roman-Gomez *et al.*, 2002
p57	2 %	Not mentioned	Not mentioned	Canalli *et al.*, 2005
	2 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
p73	20 %	Not mentioned	Not mentioned	Canalli *et al.*, 2005
	18 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
Apoptosis				
ASPP1	17 %	Decreased mRNA expression	Reduced DFS (p=0.006)	Agirre et al., 2006
DAPK	10 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
TMS1	10 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
Apaf-1	35 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]

Gene	Methylation frequency	Hypermethylation in correlation with expression in clinical samples	Correlation with clinical parameter in childhood ALL	References
Tissue invasion and metastasis				
E-cadherin	32 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
H-cadherin	32 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
ER	86 %	Not mentioned	Not mentioned	Issa *et al.*, 1996
Transcription Factors and Metabolic Enzymes				
IL-12R2	100 %	Decreased mRNA expression	Not mentioned	Airoldi *et al.*, 2006
CALC1	39 %	Not mentioned	Reduced DFS (p<0.00001)	Roman-Gomez *et al.*, 2003
Putative Tumor Suppressor Genes				
PTEN	20 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
NES1	39 %	Decreased mRNA expression	Decreased DFS (p=0.005)	Roman-Gomez *et al.*, 2004*
LATS1	30 %	Decreased mRNA expression	Decreased DFS (p=0.0005) and OS (p=0.0003)	Jimenez-Velasco *et al.*, 2004
Dkk-3	32 %	Decreased mRNA and protein expression	Not statistically significant	Roman-Gomez *et al.*, 2004#
Parkin	30 %	Not mentioned	No gene specific or childhood ALL correlated with DFS and OS were calculated	Roman-Gomez *et al.*, 2004[+]
FHIT	27.4 %	Decreased mRNA and protein expression	Not statistically significant	Zheng *et al.*, 2003
	20 %	Not mentioned	Not mentioned	Yang *et al.*, 2006
Chromosome Instability				
NNAT	69 %	Decreased mRNA expression	Not mentioned	Kuerbitz *et al.*, 2002

Roman-Gomez *et al.*, 2004 :

+ Roman-Gomez J, Jimenez-Velasco A, Castillejo JA, Agirre X, Barrios M, Navarro G, Molina FJ, Calasanz MJ, Prosper F, Heiniger A, Torres A. (2004). Promoter hypermethylation of cancer-related genes: a strong independent prognostic factor in acute lymphoblastic leukemia. *Blood.* **104(8):**2492-8.

Roman-Gomez J, Jimenez-Velasco A, Agirre X, Castillejo JA, Navarro G, Barrios M, Andreu EJ, Prosper F, Heiniger A, Torres A. (2003). Transcriptional silencing of the Dickkopfs-3 (Dkk-3) gene by CpG hypermethylation in acute lymphoblastic leukaemia. *Br J Cancer.* **91(4):**707-13.

* Roman-Gomez J, Jimenez-Velasco A, Agirre X, Castillejo JA, Barrios M, Andreu EJ, Prosper F, Heiniger A, Torres A. (2004). The normal epithelial cell-specific 1 (NES1) gene, a candidate tumor suppressor gene on chromosome 19q13.3-4, is downregulated by hypermethylation in acute lymphoblastic leukemia. *Leukemia.* **18(2):**362-5.

2.2. Methodologies in DNA Methylation Analysis

DNA methylation status of various genes have been investigated in childhood ALL (Table 2.1). These studies, however, were based on prior knowledge of target genes previously found to be aberrantly methylated in other types of cancers. Such investigations have implicated the hypothesis: hematological malignancies are arisen through leukemic-specific promoter hypermethylation. Hence, genome-wide screening of DNA methylation pattern was introduced. This platform includes restriction landmark genomic scanning (RLGS) (Kawai *et al.*, 1993), methylation-sensitive representational difference analysis (MS-RDA) (Ushijima *et al.*, 1997), methylation-sensitive restriction fingerprinting (MS-RF) (Davies, 2002), demethylating agent cDNA microarray (Suzuki *et al.*, 2002) and methylation-sensitive arbitrarily primed PCR (MS-AP PCR) (Gonzalgo *et al.*, 1997).

2.2.1. Principle of Methylation-sensitive Arbitrarily Primed PCR (MS-AP PCR)

MS-AP PCR, like other genome-wide screening techniques, has the advantage of requiring no prior knowledge of sequences of candidate genes so that it allows the investigation of genes other than those known or well characterized genes. It was developed at 1997 by a US research group (Gonzalgo *et al.*, 1997).

The principle of MS-AP PCR is illustrated in Figure 2.1. Restriction enzymes

commonly used in the MS-AP PCR are: *RsaI*, *HpaII* and *MspI*. *RsaI* is used to generate small-sized DNA fragment from genomic DNA before the digestion by methylation sensitive or insensitive restriction enzymes. *HpaII* and *MspI* both recognize the same 5'-CCGG-3' sequence. However, *HpaII* is methylation-sensitive restriction enzyme which only digests unmethylated sequence. *MspI*, on the other hand, is a methylation-insensitive restriction enzyme which digests both the methylated and unmethylated sequences. *MspI* digestion panel serves as an internal control to examine whether the DNA fragment observed under H panel is purely due to cytosine methylation of 5'-CCGG-3' sequence. After restriction digestion, arbitrarily primed PCR are done on digested DNA fragments by priming with arbitrary CG rich primers. These primers preferentially bind to CG-rich sequences in the genome.

The resulting PCR products are then analyzed by high resolution polyacrylamide gel. The PCR products are selected for further analysis when bands are observed under R panel but not under M panel. There are four possible valid DNA methylation patterns generated by the MS-AP PCR (Figure 2.2) and the corresponding gel band is excised for further analysis when the sequence displays differential methylation.

Figure 2. 1 *A schematic diagram showing the principle of MS-AP PCR.*

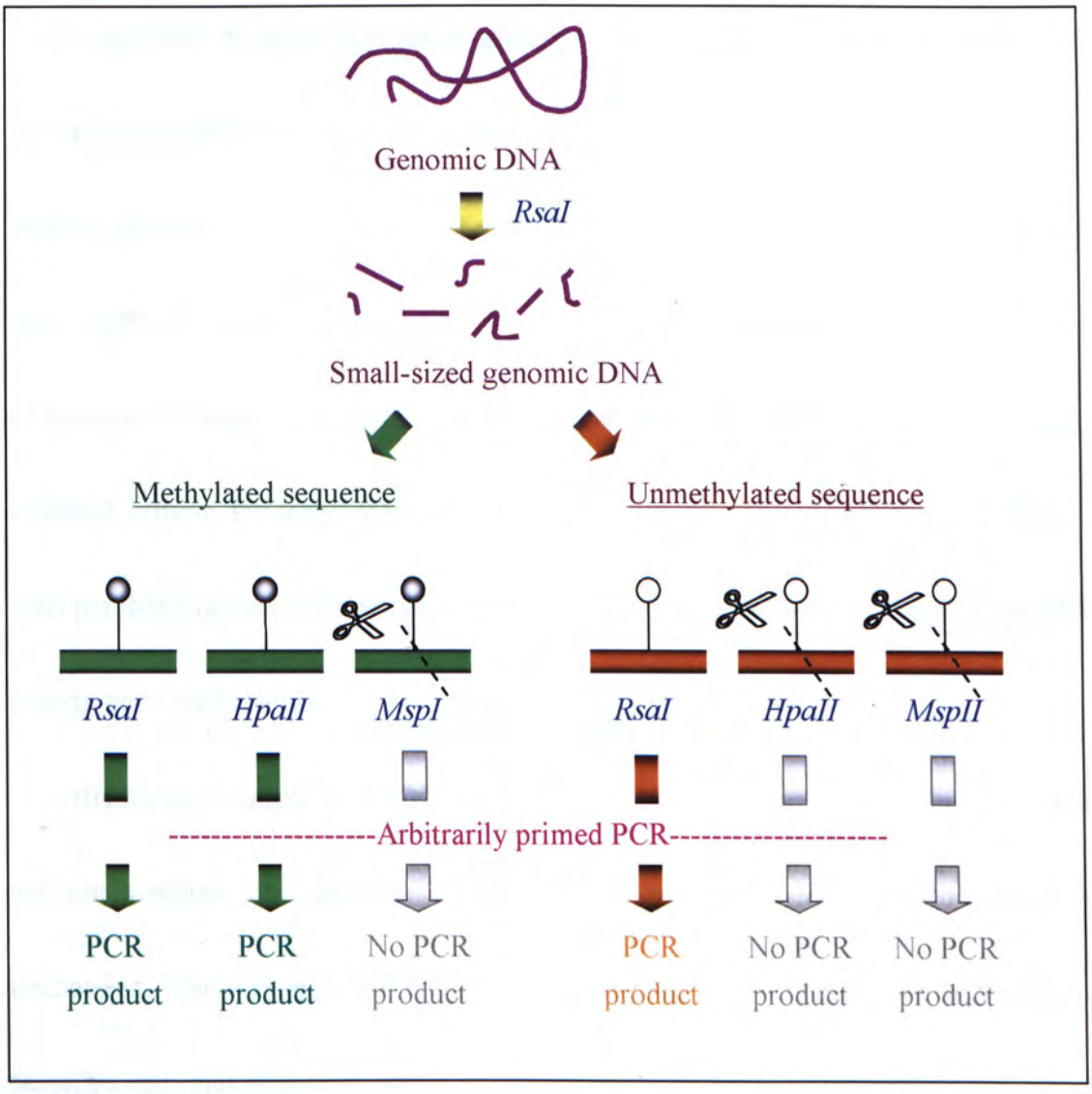
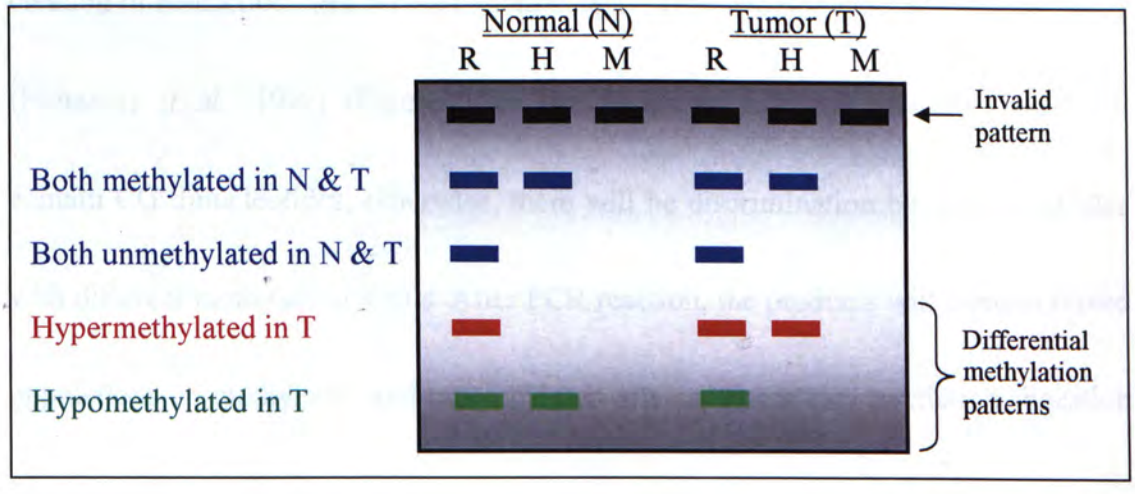


Figure 2. 2 *Possible methylation patterns generated by MS-AP PCR.*



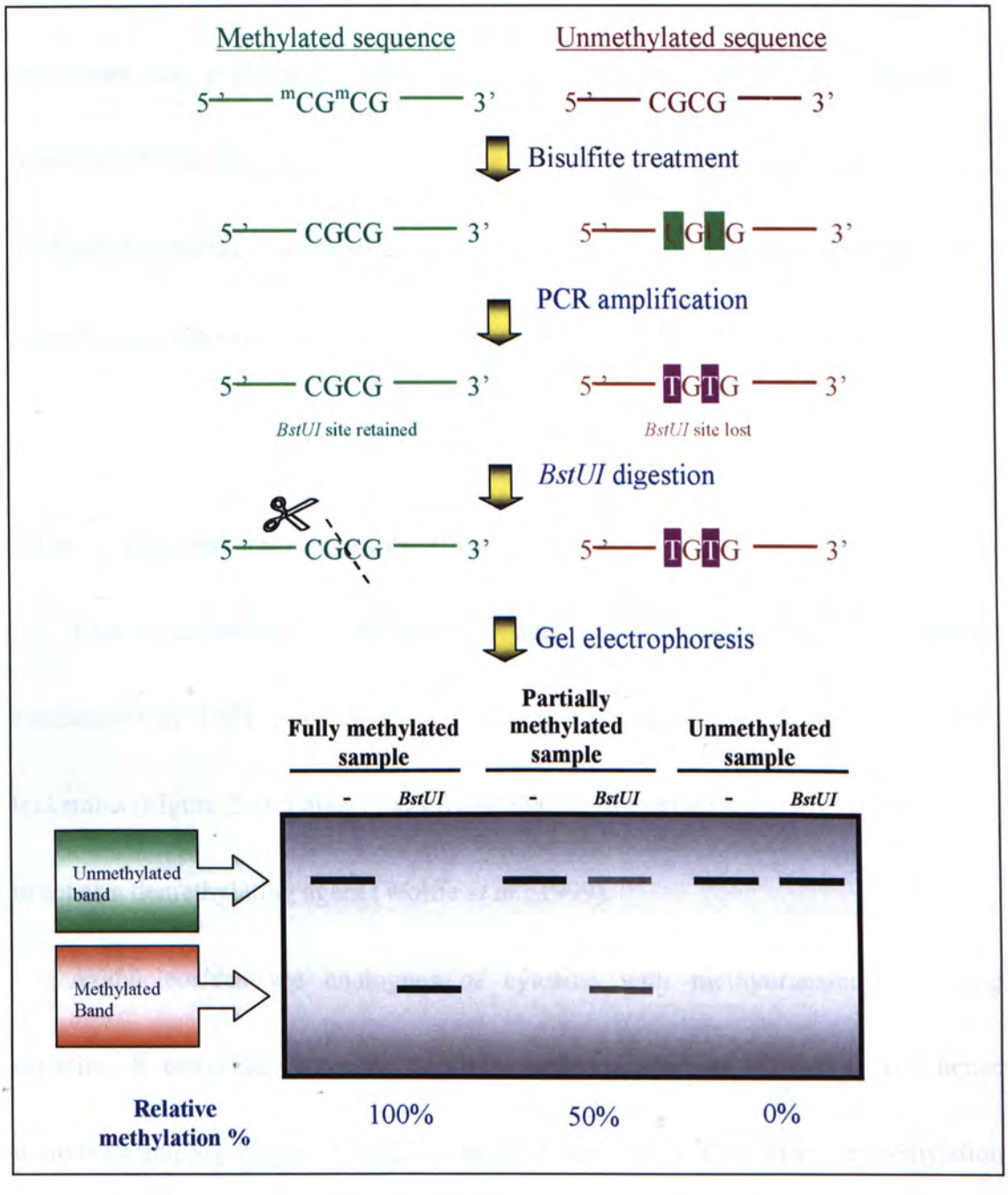
2.2.2. *Combined Bisulfite Restriction Analysis (COBRA)*

Combined bisulfite restriction analysis (COBRA) is a sensitive analysis to determine the DNA methylation status at specific gene loci by using small amount of starting genomic DNA (Xiong and Laird, 1997). When compared to other commonly used methods, such as methylation-specific PCR (MS-PCR), COBRA has the advantage of being quantitative in nature. Moreover, COBRA satisfies the three essential criteria for large scale studies: (1) quantitative accuracy, (2) compatibility with paraffin embedded tissue section and (3) Applicable to large number of samples (Xiong and Laird, 1997).

Bisulfite treatment is a DNA modification technique converting unmethylated cytosine residues into thymine residues. In contrast, methylated cytosine remains unchanged. The principle of COBRA is based on the properties of methylation sensitive enzymes that preferentially cut modified DNA which are methylated rather than unmethylated (Sadri and Hornsby, 1996). This sequence change leads to the creation of restriction sites for methylation sensitive restriction enzyme such as *BstUI* (Frommer *et al.*, 1992) (Figure 2.3). The design of COBRA primers should not contain CG dinucleotides; otherwise, there will be discrimination between templates with different methylation status. After PCR reaction, the products will contain mixed populations of methylated and unmethylated alleles. Therefore, restriction digestion

by methylation-sensitive restriction enzymes can directly reflect the methylation percentage in the original genomic DNA. The level of methylation at specific loci can be estimated from the relative intensity of the methylated and unmethylated band.

Figure 2. 3 *A schematic diagram presenting the principle of COBRA.*



2.2.3. *Cloned Bisulfite Sequencing*

Cloned bisulfite sequencing is a gene specific methylation analysis based on sequencing of PCR product amplified from bisulfite treated genomic DNA (Frommer *et al.*, 1992). It provides information on the methylation status of every cytosine residue within the target sequence. PCR products amplified by methylation primers are cloned into a plasmid vector followed by sequence analysis of a number of individual clones. The presence of cytosine in the sequence indicates the presence of a methylated cytosine, whereas the presence of thymine reflects the presence of an unmethylated cytosine.

2.2.4. *Experimental Use of Demethylating Agents*

Two azanucleosides (5-aza-cytidine and 5-aza-2'deoxycytidine [5-aza-dCR]) synthesized in 1964 were developed for cytostatic treatment primarily for acute leukemias (Figure 2.4). Later, it was found that these azanucleosides have the potency to act as a demethylating agent (Wolffe *et al.*, 1999).

Azanucleosides are analogues of cytosine with methyltransferase binding capacity. It covalently attaches to DNA-methyltransferases (DNMTs) and hence inhibits its activity (Figure 2.5) (Claus and Lubbert, 2003). Therefore, the methylation

of the newly synthesized DNA is prohibited and the methylated alleles are sequentially reduced for every DNA replication cycle, and finally lead to global demethylation (Michalowsky and Jones, 2003) (Figure 2.5).

Currently, the clinical trials of azanucleosides have been assessed in solid and hematological malignancies (Luebbert, 2000). It has been demonstrated that azanucleosides is able to inhibit growth of human tumor both in *in vitro* and *in vivo* studies. For instance, the drug efficacy of azanucleosides has been clinically studied in several myeloid neoplasias in older patients, like myelodysplasia and acute myeloid leukemia (AML) (Ruter *et al.*, 2004).

Figure 2. 4 *Chemical structures of Azanucleosides.*

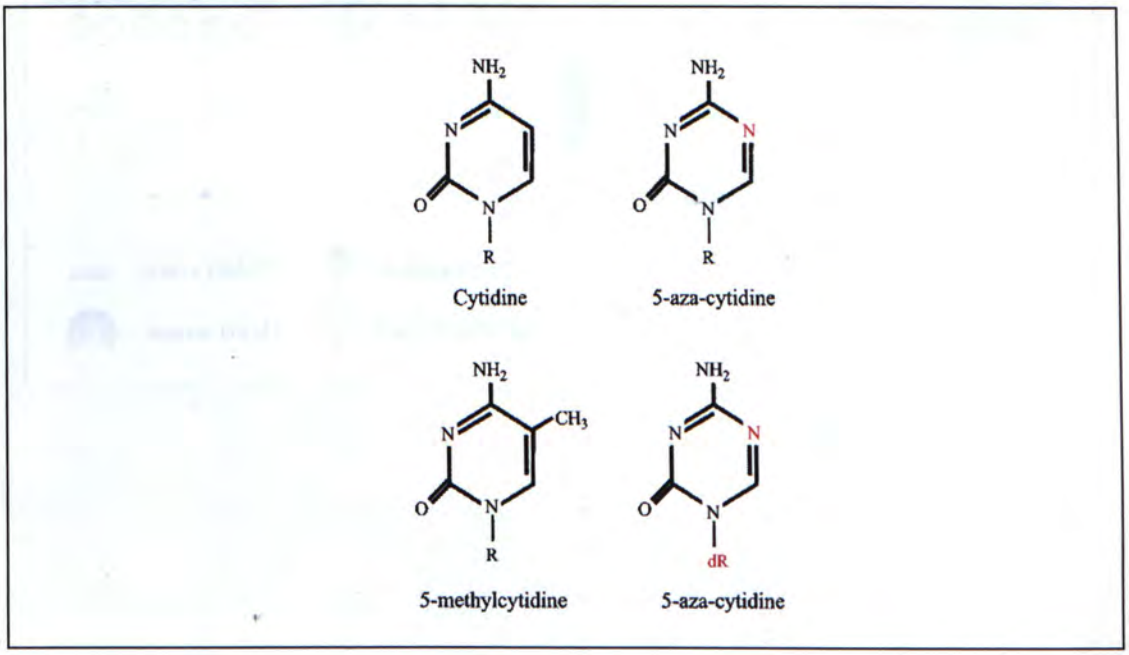
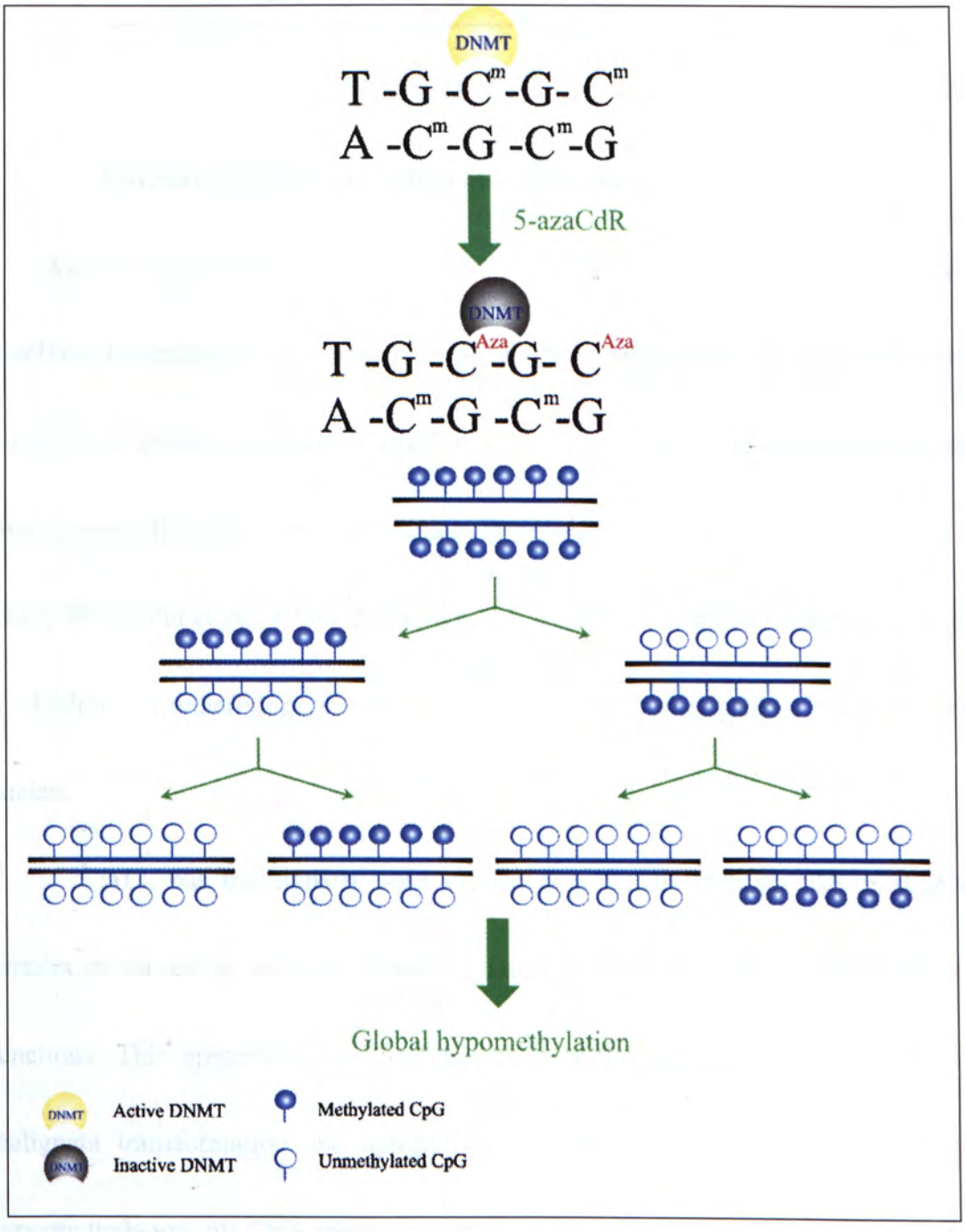


Figure 2. 5 *Demethylating action of azanucleosides.*



Chapter 3 Background of Research

3.1. Current Methylation Studies in Childhood ALL

Acute lymphoblastic leukemia (ALL) is a cancer of the hematopoietic system involving the malignant transformation of lymphoid progenitor cells which accounts for 30 % of childhood cancer (Carroll *et al.*, 2003). Dramatic advances in treatment strategy over the past few decades have increased the childhood ALL cure rate at nearly 80 % (Pui *et al.*, 1998). Although ALL is the most curable malignant diseases in children, its underlying genetic mechanism of ALL tumorigenesis remain largely unclear.

ALL has traditionally been viewed as a genetic disease, like most other cancers, is caused by progressive accumulation of malignant cells with altered gene functions. This epigenetic modification has been associated with gene silencing, malignant transformation and aging (Das and Singal, 2004). Aberrant promoter hypermethylation of CpG island is one of the epigenetic modifications of gene expression in hematological malignancies (Takahashi *et al.*, 2004). Through multi-step ALL tumorigenesis, cancer-related gene can be silenced or activated by DNA methylation. Nevertheless, the epigenetic profile of childhood ALL has not been fully studied. To date, around 100 papers have reported methylation changes in ALL,

which, however, only 40 of them focused on childhood ALL. Specifically, part of these studies focused on relapse cases rather than cases at first diagnosis and around 20 genes were investigated in childhood ALL. In addition, only few of them have demonstrated a correlation between aberrant methylation with down-regulation of the cancer-related genes and also the disease prognosis (Table 2.1). Many of these methylation studies targeted on investigating specific single-gene or multiple-genes as a profiling study. These investigations require prior information on the DNA sequence, which, however, limited the current studies to known genes or cancer-related genes that have been reported.

Researchers have also investigated the contribution of multiple-genes methylation profile in related to childhood ALL prognosis (Iravani *et al.*, 1997; Roman-Gomez *et al.*, 2004; Gutierrez *et al.*, 2003; Garcia-Manero *et al.*, 2002). These approaches select target genes that are known to play an important role in tumor suppression, cell cycle regulation, apoptosis, DNA repair or metastatic potential. Conversely, the use of genome-wide scanning approach can allow the discovery of novel candidate genes because it assesses the methylation pattern with no prior DNA sequence information and thus allow the identification of novel genes implicated in childhood ALL tumorigenesis.

3.2. Objectives of Research

To the best of our knowledge, this is the first genome-wide DNA methylation screening in ALL and childhood cancer. The objective of this study was to identify novel cancer-related genes in childhood ALL by first searching for CpG islands with leukemia-associated DNA methylation pattern using a genome-wide scanning approach, the MS-AP PCR and second by examining the role of promoter methylation in transcriptional silencing and involvement of the selected gene candidate found in both cell lines and clinical samples.

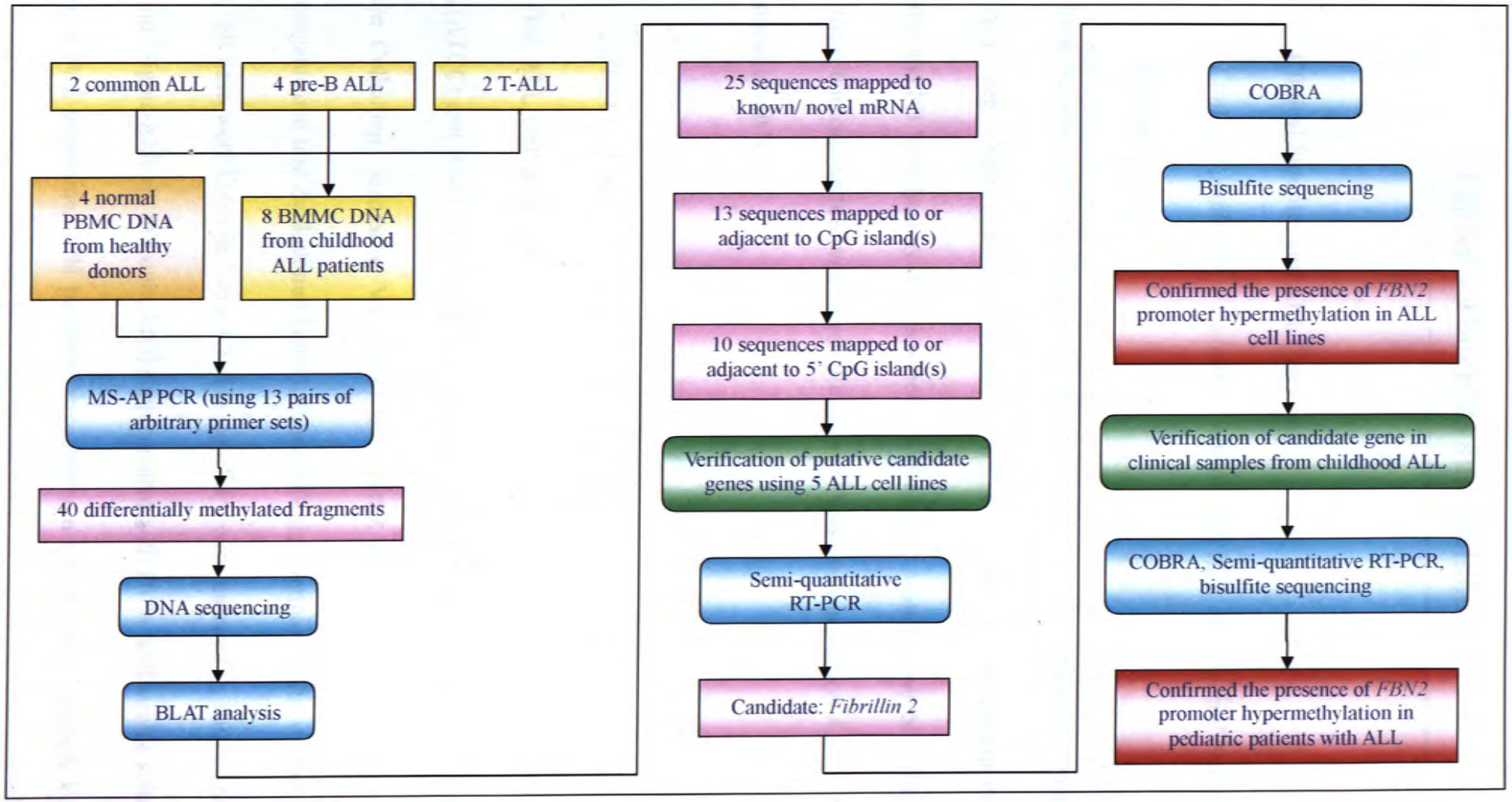
MS-AP PCR is a simple and reproducible method that has been used to identify novel cancer-related genes in tumors. For instance, this screening strategy has been employed to identify novel genes that are hypermethylated in various cancers including colon cancer, bladder cancer, pituitary adenomas and head and neck squamous cell carcinoma (Liang *et al.*, 2000; Salem *et al.*, 2000; Bahar *et al.*, 2004; Estecio *et al.*, 2006) (will be further discussed in section 6.1). These findings solidified the notion that MS-AP PCR is a feasible genome-wide methylation screening approach to identify novel cancer related genes. Consequently, the clinico-patholgical contribution of the methylation regulating candidate-gene(s) can be examined in childhood ALL. The identification of differentially methylated genes

may indicate specific molecular pathways and contribute to the prediction of prognosis in childhood ALL and finally help to improve therapeutic strategies in future.

3.3. Study Approach and Experimental Design

In this study, we employed MS-AP PCR as our platform to identify differential methylated DNA fragments from human genome using clinical samples from childhood ALL patients and normal healthy donors. Figure 3.1 summarized the experimental design of our study. The identities of target fragments identified from MS-AP PCR were then analyzed by DNA sequencing. BLAT analysis was done for each fragment to identify the presence of CpG island and to map the sequence to any known or novel genes. Methylation status of the 5' CpG island and mRNA expression of candidate genes were investigated by COBRA, cloned bisulfite sequencing and semi-quantitative RT-PCR respectively. Data from demethylation studies further elucidated the relationship between promoter hypermethylation and transcriptional silencing of gene. Finally, the involvement of aberrantly methylated gene was examined in the BM samples from pediatric patients with ALL.

Figure 3. 1 *Experimental Design of this Study.*



Chapter 4 Materials and Methods

4.1. Clinical Samples and ALL Cell Lines

4.1.1. Clinical Samples from Pediatric Patients with ALL and Normal Healthy Donors

Bone marrow (BM) aspirates and peripheral blood (PB) from pediatric patients with ALL were obtained from the Prince of Wales Hospital. Non-malignant BM aspirates and PB from healthy donors were used as control. Mononuclear cells (MC) from these clinical samples were freshly extracted (illustrated at section 4.2.1) and then stored at -80 °C

4.1.2. ALL Cell Lines

Five ALL cell lines [697 (DMSZ), CCRF-CEM (ATCC), MOLT-3 (ATCC), REH (ATCC) and Rs4;11 (ATCC)] were obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA) and DMSZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The origins of these cell lines were listed in Table 4.1. The cell lines were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 20 % fetal bovine serum (FBS), 1 % L-glutamine, 1 % penicillin/streptomyhcin (Invitrogen, Carlsbad, CA,

USA) and incubated in 5 % CO_2 at 37 ℃.

Table 4. 1 *Origins of ALL cell-lines.*

Cell lines	Origins
697	Human B-cell precursor leukemia
CCRF-CEM	Human T-cell leukemia
MOLT3	Human T-cell leukemia
REH	Human B-cell precursor leukemia
Rs4;11	Human B-cell precursor leukemia

4.2. Genomic DNA Isolation from Clinical Samples and Cell Lines

4.2.1. Ficoll Gradient Centrifugation

MC from BM and PB were isolated using Ficoll-Paque™ PLUS (Amersham Biosciences, Piscataway, NJ, USA). Briefly, the samples were diluted with 1X phosphate buffer saline (PBS) and layered gently onto an equal volume of Ficoll-Paque™ PLUS. After non-braking centrifugation at 1400 rpm for 30 minutes (mins), mononuclear cells were collected at the interphase layer and then washed twice with 1X PBS. The cells were stored at -80 ℃.

4.2.2. DNA Extraction

BMMC, PBMC and cultured cell lines were digested with 3 ml saline Tris-ethylenediamine tetraacetic acetic (Tris-EDTA) buffer containing 5 % sodium

dodecyl sulfate (SDS), 0.2 mg/ml proteinase K and incubated overnight at 50 ℃. After overnight protein digestion, equal volume of phenol/ chloroform/ isoamyl alcohol (25:24:1) (Amersham Biosciences) was added to the mixture and then shaken gently for 20 mins. After centrifugation at 3500 rpm for 30 mins, the upper aqueous layer was collected. DNA was precipitated by the addition of 1/10 volumes of 3 M sodium acetate (pH 5.2) followed by 2.2 volumes of cold 100 % ethanol (BDH Inc., Toronto, US). After overnight precipitation at -20 ℃, the mixture was centrifuged at 3500 rpm for 30 mins at 4 ℃ and washed twice with cold 70 % ethanol. The DNA pellet was air dried and dissolved in sterilized water. The quality and quantity of DNA were determined by measuring the absorbance at 260/280 nm using spectrophotometer. The extracted DNA was stored at -20 ℃.

4.3. MS-AP PCR

4.3.1. Methylation-sensitive Restriction Enzyme Digestion of Genomic DNA

Totally, 8 BMMC DNA from pediatric patients with ALL were used in MS-AP PCR and 4 PBMC DNA from normal healthy donors were recruited correspondingly. The methylation-sensitive restriction digestion was carried out using 1 μg of DNA from clinical samples as shown in Table 4.2. BMMC and PBMC DNA were digested in a total volume of 20 μl with different panels of restriction enzymes (*RsaI*, *HpaII*,

MspI) according to Table 4.3. Fifteen units of each restriction enzyme was used and the sample were incubated for 16 hours (hrs) at 37℃. All restriction enzymes were purchased from New England Biolabs (NEB, Beverly, MA, USA).

Table 4. 2 *Samples used in MS-AP PCR.*

Group	Types of sample used	Numbers of sample used	
Patient group	BMMC from pediatric patients with ALL	common ALL	2
		pre B-ALL	4
		T-ALL	2
Control group	PBMC DNA from normal healthy donors	-	4

Table 4. 3 *Panels of methylation-sensitive restriction digestion.*

	Panel R	Panel H	Panel M
Combination of restriction enzymes	*RsaI*	*RsaI* and *HpaII*	*RsaI* and *MspI*

4.3.2. Arbitrarily Primed Polymerase Chain Reaction

The arbitrarily primed polymerase chain reaction (AP-PCR) was performed according to the methodology published by Maskl *et al.*, 2001. DNA digests were amplified with 13 primer sets, each with a combination of 2 to 3 arbitrary GC-rich primers (Table 4.4 and 4.5).

PCR reactions were performed with 50 ng DNA, GeneAmp 1X Buffer II

(Applied Biosystems, Foster City, CA, USA), 1.5 mM $MgCl_2$ (Applied Biosystems), 200 μM dNTP (Amersham Bioscience), 10 pmol of each primers, 0.5 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 4 μCi of α^{33}P-labeled dATP (Perkin Elmer, Foster City, CA, USA) in a final volume of 10 μl.

The reaction mixtures were then subjected to 95 ℃ hot start for 10 mins, followed by 42 cycles of amplification (94 ℃, 2 mins; 40 ℃, 1 min ; 72 ℃, 2 mins) with a final extension at 72 ℃ for 10 mins.

The PCR products were mixed with equal volume of formamide dye buffer [95 % formamide, 20 mM EDTA (pH 8.0), 10 mM NaOH, 0.05 % bromphenol blue and 0.05 % xylene cyanol], denatured at 95 ℃ and then cooled immediately on ice. Samples (2.5 μl) were resolved on 5 % 7.5 M urea denaturing polyacrylamide gel for 90 mins at constant voltage of 1700 V, 300 mA and 110 W. After electrophoresis, the gel was dried at 80 ℃ for 3 hrs and exposed to BioMax MR autoradiographic film (Eastman Kodak Company, NY, USA) with an intensifying screen at -80℃ overnight.

Table 4. 4 *Primer Sequences for MS-AP PCR.*

Primer	Primer sequences
G1	5'-GCG CCG ACG T-3'
G2	5'-CGG GAC GCG A-3'
G3	5'-CCG CGA TCG C-3'
G4	5'-TGG CCG CCG A-3'
G5	5'-TGC GAC GCC G-3'
GC1	5'-GGG CCG CGG C-3'
GC2	5'-CCC CGC GGG G-3'
GC4	5'-GCG CGC CGC G-3'
GC5	5'-GCG GGG CGG C-3'
TIM1	5'-AGC GGC CGC G-3'
TIM7	5'-GAG GTG CGC G-3'
TIM10	5'-AGG GGA CGC G-3'
TIM11	5'-GAG AGG CGC G-3'
TIM12	5'-GCC CCC GCG A-3'
TIM13	5'-CGG GGC GCG A-3'
TIM17	5'-GGG GAC GCG A-3'
TIM18	5'-ACC CCA CCC G-3'

Table 4. 5 *Primer sets for MS-AP PCR.*

Primer set number	Primer combination
1	G2, G4, G5
2	GC1, GC4
3	GC1, GC5
4	GC2, GC5
5	TIM1, TIM7
6	TIM1, TIM18
7	TIM11, TIM12, TIM13
8	TIM13, TIM17
9	TIM1, TIM13
10	G1, G3, G4
11	G1, G4, G5
12	G1, G2, G3
13	G4, GC4, TIM10

4.3.3. Isolation of Differentially Methylated DNA Fragments

Differentially methylated DNA fragments were identified by comparing the pattern of the 3 restriction digestion panels. Target bands were isolated from the gel and placed in 1.5 ml microcentrifuge tube containing 40 µl sterile water. The tubes were incubated at 60 ℃ overnight to facilitate the dissolution of DNA fragment. Eluted DNA (1.5 µl) was re-amplified with the same primer set used in the MS AP-PCR to generate sufficient template for cloning and DNA sequencing.

PCR re-amplification mix contained 1X PCR buffer [10 mM Tris-HCL (pH 9.0), 1.5 mM $MgCl_2$ and 50 mM KCL], 200 µM dNTP (Amersham Biosciences), 12.5 pmol of each primer, 5 % dimethyl sulfoxide (DMSO) and 1 unit of *Taq* DNA polymerase (Amersham Biosciences) in a total volume of 25 µl. The reaction mixture was then subjected to 95 ℃ hot start for 2 mins, followed by 45 cycles of amplification (94 ℃, 1 mins; 49 ℃, 45 seconds (s) ; 72 ℃, 45 s) with a final extension of 72 ℃ for 10 mins. The PCR products were analyzed by 2 % (weight/ volume) agarose gel with 0.5 µg/ml ethidium bromide in 0.5X TBE buffer (44.5 mM Tris-base, 49 mM boric acid, 1 mM EDTA). The target PCR fragments were purified by the Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol and eluted in 30 µl sterile water.

4.4. Cloning of Differentially Methylated DNA Fragments

4.4.1. TA Cloning

The eluted PCR products were cloned using the TA Cloning® Kit (Invitrogen) according to manufacture's recommendations. The fragments were ligated with pCR2.1 vector (12.5 ng) in the presence of 1X ligation buffer and 1 Weiss units of T4 DNA ligase. The final reaction volume was 5 μl and the ligation mixtures were incubated at 14 ℃ overnight.

Transformation was performed by using TOP10 competent cell and 50μl of cells were thawed on ice followed by the addition of 2 μl of ligated product. After incubation on ice for 30 mins, the cells were heat shock at 42 ℃ for 30 s and then immediately kept on ice. S.O.C medium (250μl) was added to the cells and the vial was shaken horizontally at 225 rpm at 37 ℃ for 1 hr. Upon incubation, the cell mixture was spinned down at 6000 rpm for 15 s and 200 μl of medium was removed to minimize the total volume of cells suspension. The remaining cell mixture was resuspended and spread on LB agar plate [1 % tryptone, 0.5 % yeast extract, 1 % NaCl, 15 % agar (pH 7.0) with 0.1 mg/ml ampicillin and 0.8 mg X-gal] and incubated at 37 ℃ overnight to allow blue/white colony selection.

4.4.2. Screening of Positive Clones

Eight white bacterial colonies were picked from each LBA agar plates and resuspended in 4 µl of sterilized water which acted as the PCR template. Cloned direct PCR was conducted to ensure the proper size of insert. PCR reaction was contained GeneAmp 1X Buffer II, 1.5 mM $MgCl_2$, 200 µM dNTP 10 pmol of each primer (T7 promoter and M13 reverse primers) (Table 4.6) and 0.5 units of AmpliTaq Gold DNA polymerase in a final volume of 20 µl. The reaction mixture was then subjected to 95 ℃ hot start for 10 mins, followed by 35 cycles of amplification (94 ℃, 2 mins; 40 ℃, 1 min ; 72 ℃, 2 mins) with a final extension of 72 ℃ for 10 mins. The PCR products were then analyzed by 2 % (w/v) agarose gel.

Table 4. 6 *Primer sequences and PCR conditions for DNA sequencing, COBRA and semi-quantitative RT-PCR.*

Amplicon	Purpose	Primer sequence	Tm (℃)	PCR product size (bp)
T7 promoter M13 reverse	DNA Sequencing	5'-TAATACGACTCACTATAG- 3' 5'-AGGAAACAGCTATGACCA-3'	50	N/A
FBN2	COBRA	Forward: 5'-TTTTTGTTTGTTTGTTTGTTTTTTTT-3' Reverse: 5'-AAAATCAAAATCTAATAAACCCTTC-3'	55	404
FBN2	Semi-quantitative RT-PCR	Forward: 5'-GTTGCAGGTAAAGACGTGTG-3' Reverse: 5'-CTCACAGAACTCTCGGGTCC-3'	60	590
GAPDH	Semi-quantitative RT-PCR	Forward: 5-'TCTAGACGGCAGGTCAGGTCCACC-3' Reverse: 5'-CCACCCATGGCAAATTCCATGGCA-3'	60	598

4.4.3. Preparation of Plasmid DNA by Alkaline Lysis Method

Bacterial colonies with positive clones were inoculated in 1.5 ml LB Broth (Amersham Biosciences) with 0.1 mg/ml ampicillin and shaken at 225 rpm for 16 hrs at 37 ℃ . Cell suspensions were centrifuged at maximum speed for 20 s to remove the LB Broth. The cell pellet were resuspended in 100 μl GTE buffer [50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA] and incubated at room temperature (RT) for 5 mins to lyse bacterial membrane. Bacterial protein, chromosomal DNA and plasmid DNA were denatured by addition of 100 μl freshly prepared 0.2 M NaOH / 1 % SDS solution. The mixture was inverted upside down and placed at RT for 5 mins and then neutralized with 150 μl of 5M potassium acetate (pH 4.8). The mixture was inverted upside down and placed on-ice for another 5 mins. The mixture was centrifuged at maximum speed for 10 mins at 4 ℃ and the supernatant was collected. Plasmid DNA was precipitated by the addition of 2.2 volume of cold absolute ethanol and the pellet was washed twice by 70 % ethanol. The pellet was air dried and resuspended in 50 μl sterilized water.

4.5. DNA Sequence Analysis of Differentially Methylated DNA Fragments

4.5.1. Dye-terminator Cycle Sequencing

Dye-terminator cycle sequencing was performed on cloned target DNA

product by BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacture's instructions. The sequencing reaction was performed with 1 μg plasmid DNA, 1X Bigdye® v3.1 Sequencing Buffer, 1X ready reaction premix and 10 pmol of M13 reverse primer (Table 4.6). The reaction mixture was then subjected to 96 ℃ initial denaturation for 1 mins followed by 30 cycles of dye-termination reaction (96 ℃, 10 s; 50 ℃, 5 s ; 60 ℃, 4 s). The sequencing products were purified by Sephadex G-50 spin column (Amersham Biosciences). The purified products were mixed with an equal volume of Hi-Di™ Formamide (Applied Biosystems) followed by denaturation at 95 ℃ for 3 mins. The electrophoresis was performed by using ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Sequence homologies were determined using the blastn program of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

4.5.2. CpG islands Analysis of Differentially Methylated Sequences

The differentially methylated fragment was verified by the presence of *HpaII* restriction site. The sequences were analyzed by the Human BLAT Search provided from the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/). The sequences were searched against putative CpG islands, which are defined as a DNA

region(s) greater than 200bp with GC content higher than 0.5 and an observed/expected CpG ratio above 0.6 (Gardiner-Garden and Frommer, 1987).

4.6. DNA Methylation Analysis

4.6.1. Sodium Bisulfite Modification

The sodium bisulfite modification of genomic DNA was performed by the EZ DNA modification Kit (Zymo Research, Hornby, Canada) according to manufacturer's instruction. Firstly, 2 μg of genomic DNA was mixed with 5 μl of M-Dilution Buffer and finally adjusted to 50 μl by sterilized water. The mixture was incubated at 37 ℃ for 15 mins. CT Conversion Reagent is a solid chemical mixture and it was prepared by addition of 750 μl sterilized water together with 210 μl M-Dilution Buffer. After 37 ℃ incubation, 100 μl of the freshly prepared CT Conversion Reagent was added to the mixture followed by gentle vortexing. The mixture was then incubated in dark at 50 ℃ for 16 hrs. After overnight incubation, the sample was incubated on ice for 10 mins. M-Binding Buffer (400 μl) was added to the mixture and the mixture was loaded into Zymo-Spin I Column. The column was centrifuged briefly to allow DNA collection and it was washed by 200 μl of M-Wash Buffer, M-Desulphonation Buffer and M-Wash Buffer sequentially. Finally, the modified DNA were eluted by 30 μl sterilized water and stored at -20 ℃.

4.6.2. Combined Bisulfite Restriction Analysis

COBRA was used to determine the methylation level of specific locus. Ten BMMC and 10 PBMC from non-malignant donors were used as control. Besides, 5 ALL cell lines and 64 BMMC from pediatric patients with ALL samples were use d in COBRA (Table 4.7). The desired locus was amplified from bisulfite modified DNA with specific primers which were designed by the MethPrimer Program (http://www.urogene.org/methprimer/index1.html) (Table 4.6). PCR reactions were performed with 3 μl bisulfite modified DNA template, GeneAmp 1X Buffer II, 1.5 mM $MgCl_2$, 200 μM dNTP, 10 pmol of each primer and 0.5 unit of AmpliTaq Gold DNA polymerase in a final volume of 20 μl. The reaction mixture was then subjected 40 cycles of amplification and the PCR conditions were shown in Table 4.6. The PCR products were digested with *BstUI* (NEB) at 60 ℃ overnight and the products were resolved by non-denaturing 10 % polyacrylamide gel. The gel was stained with 0.5 μg/ml ethidium bromide in 1X TBE buffer and visualized under UV illuminator.

Table 4. 7 *Samples used in COBRA.*

Group	Types of sample used	Numbers of sample used	
ALL cell lines	697, CCRF-CEM, MOLT3, REH, Rs4;11	6	
Patient group	BMMC from pediatric patients with ALL	Pro-B ALL	1
		Early pre-B ALL	5
		Common ALL	35
		Pre-B ALL	17
		T-ALL	6
Control group	Non-malignant BMMC	10	
	PBMC form normal healthy donors	10	

4.6.3. Cloned Bisulfite Genomic Sequencing

The remaining un-digested PCR products amplified from bisulfite modified genomic DNA were cloned into the pCR2.1 vector using the TA Cloning® Kit as described previously in section 4.4.1. The plasmid DNA was prepared by alkaline lysis method as mentioned in section 4.4.3 and sequenced by ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) to identify the methylation `status of each CG site among specific locus.

4.7 Gene Expression Study

4.7.1. RNA Extraction from Clinical Samples and ALL Cell Lines

Total RNA was extracted by resuspending the BMMC, PBMC and ALL cell lines in TRIzol® Reagent (Invitrogen). The cells were completely homogenized in 1 ml

TRIzol® Reagnet and then incubated for 10 mins at RT. Chloroform (200 μl) (BDH Inc.) was added to each sample and the mixture was shaken vigorously for 15 s. It was incubated for 5 mins at RT and then centrifuged at 12000 rpm for 15 mins at 4 ℃. The upper aqueous layer was collected followed by the addition of 500 μl isopropyl alcohol (BDH Inc.). The mixture was mixed gently and incubated for 30 mins at -20 ℃. After incubation, the mixture was centrifuged at 12000 rpm for 20 mins at 4 ℃. The RNA pellet was washed twice with 75 % ethanol. Finally, the pellet was air dried and dissolved in DEPC-treated water. DNA contaminations were eliminated by DNaseI digestion (Amersham Biosciences). Total RNA was treated with TRIzol® Reagent again to remove the DNaseI, the procedures were repeated once. The quality and quantity of RNA were determined by measuring absorbance at 260/280nm with a UV spectrophotometer.

4.7.2. Reverse Transcription PCR

One microgram of total RNA was used to synthesize first strand cDNA by using the MuLV reverse transcriptase (Applied Biosystems). The total reaction volume was 20 μl containing 50 units of MuLV reverse transcriptase, 50 pmol random hexamer (Applied Biosystems), 20 units RNase inhibitor (Applied Biosystems), GeneAmp 1X Buffer II, 1.5 mM $MgCl_2$, 200 μM dNTP. The conditions for synthesis were: 22 ℃

for 25 mins, 42 ℃ for 65 mins and 92 ℃ for 2 mins.

4.7.3. Semi-quantitative RT-PCR

In order to determine the relative expression of the target gene, semi-quantitative PCR was performed. Primers specific for each target genes were designed by the Primer3 program developed by Whitehead Institute for Biomedical Research (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Totally 27 BMMC samples from pediatric patients with ALL were used in semi-quantitative RT-PCR (Table 3.8). Briefly, 1 μl of the first strand cDNA was used for PCR amplification in a total volume of 20 μl. The reaction mixture contained 1X GeneAmp Buffer II, 1.5 mM $MgCl_2$, 200 μM dNTP, 10 pmol of each primer (*GAPDH* and target genes) (Table 3.7) and 0.5 units of AmpliTaq Gold DNA polymerase. The reaction mixtures were then subjected to 95 ℃ hot start for 10 mins, followed by 30 cycles of amplification according to Table 3.7 with a final extension of 72 ℃ for 10 mins. The PCR products were then analyzed by 2 % (w/v) agarose gel. The relative expressions of the target gene were determined by the normalization with the housekeeping gene, *GAPDH*, which served as an internal control.

Table 4. 8 *Samples used in semi-quantitative RT-PCR.*

Group	Types of sample used	Numbers of sample used	
ALL cell lines	697, CCRF-CEM, MOLT3, REH, Rs4;11	6	
Patient group	BMMC from pediatric patients with ALL	Early pre-B ALL	1
		Common ALL	14
		Pre-B ALL	7
		T-ALL	5
Control group	PBMC form normal healthy donors	13	

4.7.4. 5-aza-2'-deoxycytidine Demethylation Treatment

Cells were seeded at a proper starting density as recommended by the providers (DMSZ and ATCC). After 2 to 3 passages of high cell viability (greater than 95 %), cells were seeded at a density of $1X10^6$ in a 25 cm^3 culture flask and were treated with different concentration (1 μM, 3 μM, 5 μM, 10 μM) of 5-Aza-dCR (Sigma-Aldrich) for 4 days. The drug was replenished at 48 hrs during treatment period. At the end of the drug treatment, cells were collected for RNA extraction. The relative expressions of the target gene under different concentration of 5-Aza-dCR were determined by the normalization with the housekeeping gene, *GAPDH*, which served as an internal control.

Chapter 5 Results

5.1. Generation of DNA Methylation Pattern by MS-AP PCR

A total of 8 pediatric patients with acute lymphoblastic leukemia (ALL) (2 common ALL, 4 pre-B ALL and 2 T-ALL) and 4 normal healthy control subjects were recruited for MS-AP PCR. A representative MS-AP PCR gel image showing the methylation patterns generated from the bone marrow (BM) and peripheral blood (PB) mononucleated cell (MC) samples from the patients and normal controls was illustrated in Figure 5.1.

The analysis of the methylation patterns generated by MS-AP PCR was summarized in Figure 5.2. The selection criteria for identifying a differential methylation pattern were: (1) absence of band under M panel, (2) consistence of methylation status showed in all normal controls (i.e. either all 4 normal controls were methylated or unmethylated) and (3) $\geq$1 of the 8 leukemic samples showed differential pattern compared with the normal controls.

Figure 5. 1 *(A) A representative gel image of methylation pattern obtained from MS-AP PCR.*
(B) Examples of differential methylation patterns: hypermethylation and hypomethylation.

Genomic DNA from clinical samples of childhood ALL BM and normal PBMC were digested by 3 combinations of restriction enzymes (R, H and M panels as described in section 4.3.1.) and were amplified by 13 combinations of arbitrary GC-rich primers. Hypermethylation was indicated by the positive methylation (marked by arrows) in ≥1 leukemic samples, but unmethylated in all normal PBMC samples. Hypomethylation was indicated by the positive methylation status (marked by arrows) in all normal PBMC samples but unmethylated in ≥1 leukemic samples.

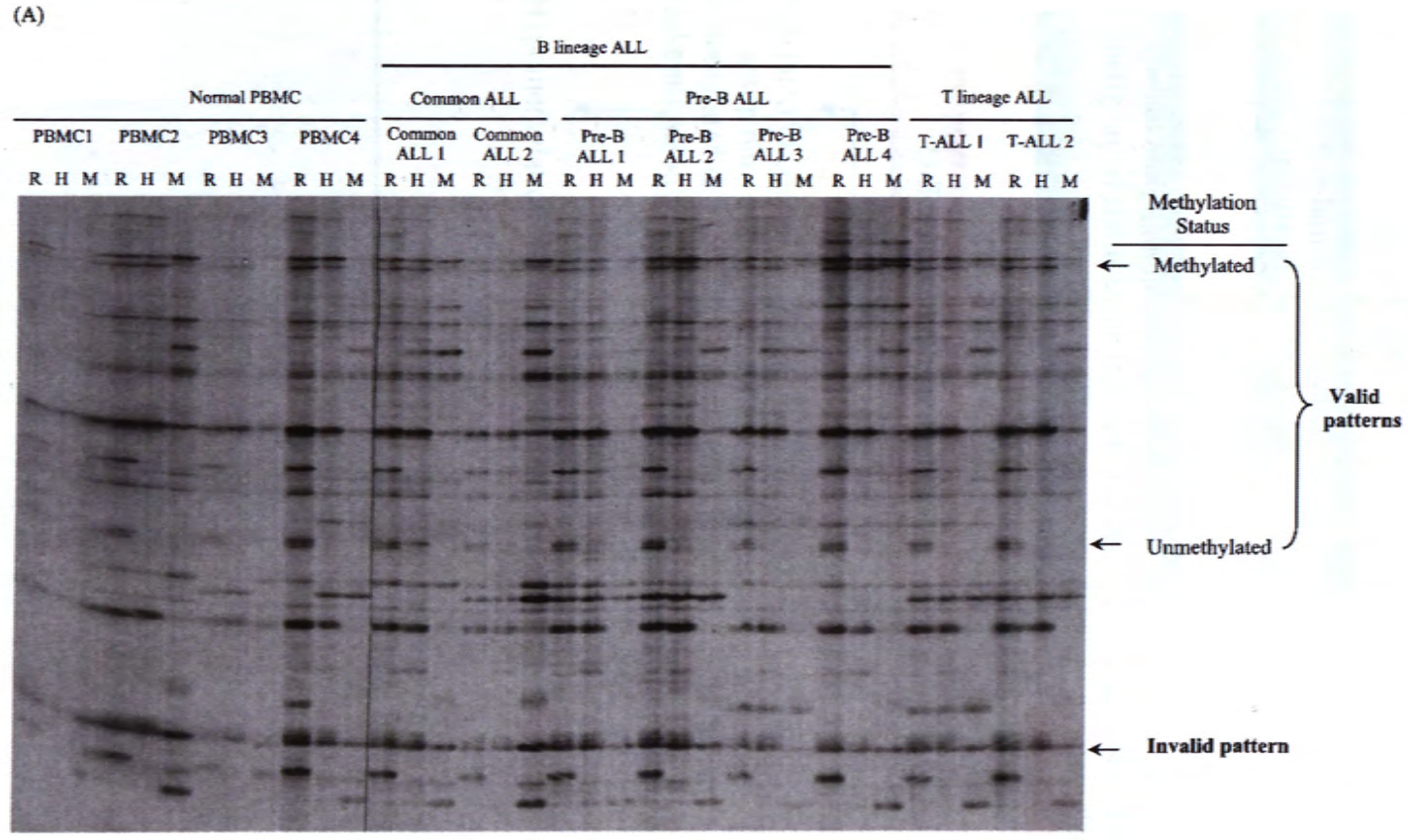
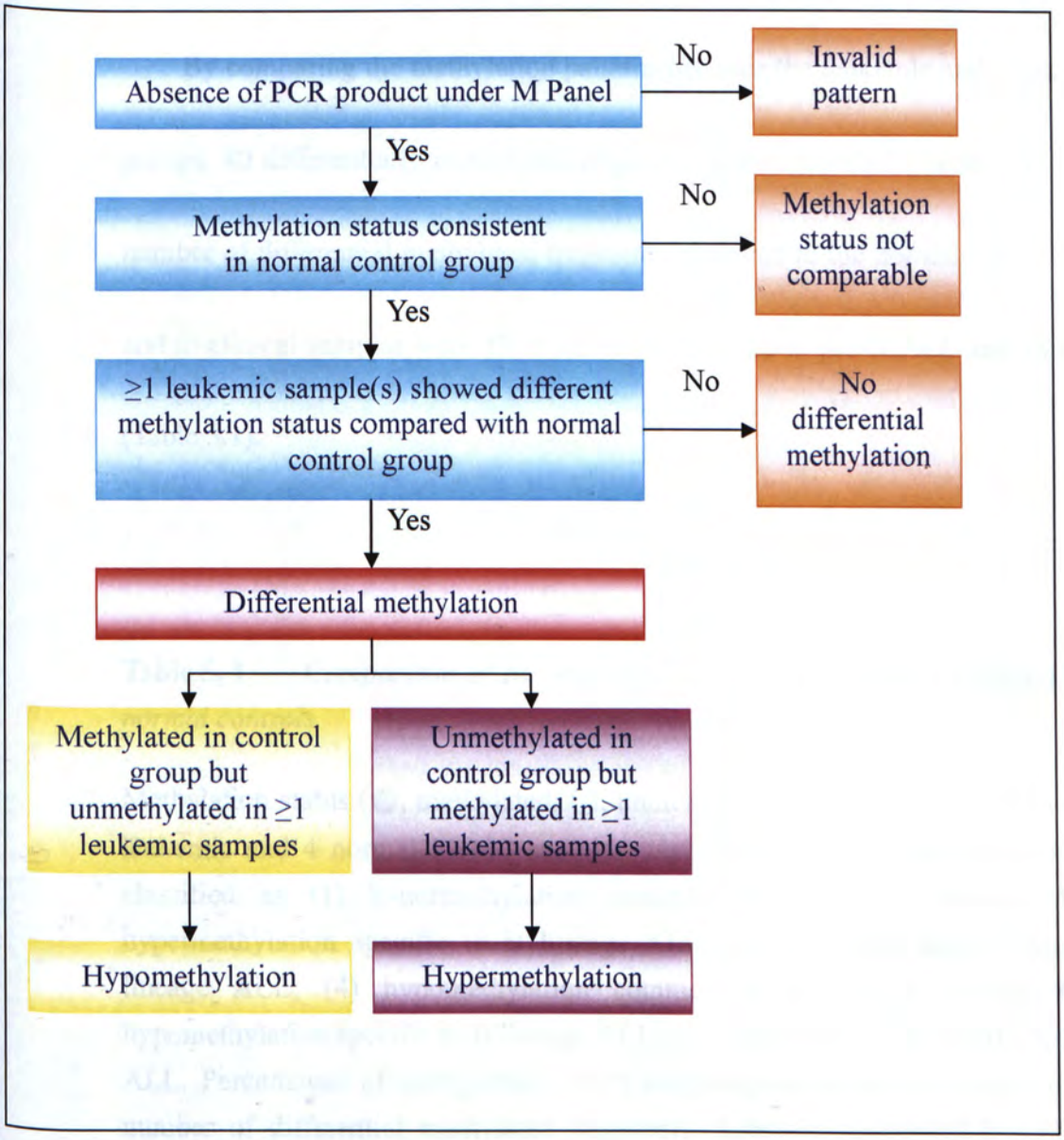

(A)
B lineage ALL
Normal PBMC
Common ALL
Pre-B ALL
T lineage ALL
PBMC1
PBMC2
PBMC3
PBMC4
Common ALL 1
Common ALL 2
Pre-B ALL 1
Pre-B ALL 2
Pre-B ALL 3
Pre-B ALL 4
T-ALL 1
T-ALL 2
R H M R H M R H M R H M R H M R H M R H M R H M R H M R H M R H M R H M
Methylation Status
Methylated
Unmethylated
Valid patterns
Invalid pattern
(B)
Normal PBMC
Childhood ALL clinical samples
R H M R H M R H M R H M R H M
Hypermethylation
Hypomethylation
Differential methylation patterns

Figure 5. 2 *Flow chart demonstrating the validation of methylation patterns.*



5.1.1. Differential Methylation Patterns of Childhood ALL

By comparing the methylation patterns between the leukemic and normal control groups, 40 differentially methylated fragments were identified (Table 5.1). The total number of differential methylated fragments observed in the normal controls were 13 and in clinical samples were 13 in common ALL, 22 in pre-B ALL and 18 in T-ALL (Table 5.1).

Table 5. 1 *Comparison of the methylation patterns between childhood ALL and normal controls.*

Methylation status (●, methylated; ○, unmethylated) of the 40 DNA sequences in 8 leukemic and 4 normal PBMC samples were shown. Their methylation status was classified as (1) hypermethylation common in B and T lineage ALL; (2) hypermethylation specific to B lineage ALL; (3) hypermethylation specific to T lineage ALL; (4) hypomethylation common in B and T lineage ALL; (5) hypomethylation specific to B lineage ALL; (6) hypomethylation specific to T lineage ALL. Percentages of methylation (% of methylation) in each sample and the total number of differential methylated fragments observed in each ALL subtype were shown at the bottom of the table. *(Total no. of methylated fragments indicates the maximum numbers of methylated fragments found in each leukemic group.)*

Seq no.	Normal PBMC				Common ALL		Pre-B ALL				T-ALL		Category
	1	2	3	4	5	6	7	8	9	10	11	12	
1	○	○	○	○	○	○	●	●	○	○	○	○	2
2	○	○	○	○	●	○	●	○	●	○	●	●	1
3	○	○	○	○	○	●	○	○	●	●	○	○	2
4	○	○	○	○	○	○	○	●	○	○	○	○	2
5	○	○	○	○	●	○	●	●	●	○	○	○	2
6	●	●	●	●	●	○	●	●	●	●	●	○	4
7	●	●	●	●	○	●	●	●	●	●	●	●	5
8	●	●	●	●	○	○	○	○	○	○	○	○	4
9	●	●	●	●	○	○	○	○	○	○	○	○	4
10	○	○	○	○	●	○	○	○	○	○	●	●	1
11	○	○	○	○	○	○	●	●	●	○	●	○	1
12	○	○	○	○	○	○	●	○	●	○	●	○	1
13	○	○	○	○	●	●	●	○	●	○	●	○	1
14	○	○	○	○	○	○	●	○	○	○	○	○	2
15	○	○	○	○	○	○	●	○	●	●	○	●	1
16	○	○	○	○	○	○	○	○	●	●	○	●	1
17	○	○	○	○	○	○	○	○	○	○	●	●	3
18	○	○	○	○	○	○	○	○	●	○	●	●	1
19	○	○	○	○	○	○	○	○	●	●	○	●	1
20	○	○	○	○	○	○	○	○	●	○	○	○	2
21	○	○	○	○	○	○	○	●	○	●	○	○	2
22	○	○	○	○	○	○	○	●	○	○	○	○	2
23	●	●	●	●	●	●	●	●	●	●	●	○	6
24	○	○	○	○	●	●	●	●	●	●	●	●	1
25	●	●	●	●	○	○	○	○	●	○	○	○	4
26	●	●	●	●	○	●	●	●	●	○	●	●	4
27	●	●	●	●	○	●	●	●	●	○	●	●	4
28	●	●	●	●	●	●	●	●	●	●	○	●	6
29	●	●	●	●	○	○	○	○	○	○	○	○	4
30	○	○	○	○	○	○	○	○	○	○	●	●	3
31	○	○	○	○	○	○	○	●	●	●	○	●	1
32	○	○	○	○	○	○	○	●	○	○	●	○	1
33	○	○	○	○	●	○	○	●	○	○	○	○	2
34	○	○	○	○	○	●	●	○	○	○	●	●	1
35	○	○	○	○	●	○	○	○	○	○	○	○	2
36	●	●	●	●	●	●	●	●	●	○	●	●	5
37	○	○	○	○	○	○	○	○	○	●	○	○	2
38	●	●	●	●	●	○	○	○	○	○	○	○	5
39	○	○	○	○	○	●	○	○	○	○	○	○	2
40	●	●	●	●	●	●	●	●	●	●	●	○	1
Total No. of methylated fragments	13				13		22				18		
% of methylated fragments	33				33	30	45	45	55	33	45	40	
Total No. of unmethylated fragments	27				27		18				22		
% of unmethylated fragments	68				68	70	55	55	45	68	55	60	

5.1.2. Methylation Patterns of B and T lineages Childhood ALL

The observed methylation statuses from MS-AP PCR were further categorized into 6 groups: (1) hypermethylation common to both B and T lineage ALL; (2) hypermethylation specific to B lineage ALL; (3) hypermethylation specific to T lineage ALL; (4) hypomethylation common to B and T lineage ALL; (5) hypomethylation specific to B lineage ALL; (6) hypomethylation specific to T lineage ALL. Table 5.2 summarizes the methylation statuses according to B or T lineages of ALL. It was found that hypermethylation (70 %) were generally more common than hypomethylation (30 %). Moreover, lineage specific hypermethylation were more prevalent with respect to B lineage (30%) than T lineage ALL (5%). In contrast, hypomethylation were similarly infrequent for both B lineage ALL (7.5%) and T lineage ALL (5%).

Table 5. 2 *Percentages of methylation statuses observed in childhood ALL.*

Characteristics	% of hypermethylation	% of hypomethylation
Common to B and T lineage ALL	35 % (14/40)	17.5 % (7/40)
B lineage ALL only	30 % (12/40)	7.5 % (3/40)
T lineage ALL only	5 % (2/40)	5 % (2/40)
Total	70 % (28/40)	30 % (12/40)

5.2. UCSC BLAT Analysis of Differential Methylated DNA Sequences

Differentially methylated DNA fragments were analyzed by DNA sequencing. To eliminate false positive, *HpaII* restriction site should be present within the DNA sequence. This indicated the fragments identified from the MS-AP PCR were solely due to differential methylation. UCSC BLAT analysis of the 40 differentially methylated DNA sequences (28 hypermethylated and 12 hypomethylated) revealed 25 of the sequences showed homology to known or novel genes. Among the 25 genes, 13 of them were adjacent to or within defined CpG island (Table 5.3).

Table 5. 3 *Characteristics of the 25 differentially methylated sequences showing sequence homology to known or novel genes.*

Sequence No.	Corresponding Known mRNA	CpG island	Location of corresponding CpG island	Chromosomal location
1	Tumor suppressor homolog 1 (Drosophila)	✓	5' end	4q35.2
5	Paired box protein 6	✓	within transcribe region	11p13
6	Chromosome 14 open reading frame 58	✓	overlapped with 5' end	14q24.3
8	Myeloid/lymphoid or mixed-lineage leukemia	✓	overlapped with 5' end	11q23.3
9	ATP-dependent RNA helicase	×	-	14q32.13
11	TAB1-like protein	×	-	22q13.1
13	SH2 and PH domain-containing adapter protein APS	✓	~2000bp away from 5' end	7q22.1
14	Hypothetical protein PSEC0266	×	-	22q13.31
16	G protein-coupled receptor kinase 5	×	-	10q26.11
18	Ataxin 2	✓	5' end	12q24.12
19	Fibrillin 2 precursor	✓	overlapped with 5' end	5q23.1
20	SFRS protein kinase 2	×	-	7q22.3
22	Hypothetical protein FLJ35924	×	-	18p11.2
23	Zinc fingers and homeoboxes protein 2	✓	overlapped with 5' end	12q13.11
24	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2	✓	~ 700bp away from 3' end	12q21.31
25	Striatin, calmodulin binding protein 4	×	-	19q13.32
26	Sarcosine dehydrogenase	×	-	9q34.2
27	Zinc finger, BED domain containing 4	✓	overlapped with 5' end	22q13.33
28	Sushi-repeat-containing protein, X-linked	✓	3' end	Xp11.4
29	Transcription factor 20 (AR1) (TCF20)	×	-	22q13.2
32	KIAA0518 protein	×	-	15q15.1
33	RAN binding protein 1	✓	overlapped with 5' end	22q11.21
34	DNA (cytosine-5-)-methyltransferase 3 beta	×	-	11.21
39	Pleckstrin and Sec7 domain containing protein 3	×	-	8p22
40	Platelet-derived growth factor receptor, alpha polypeptide	✓	overlapped with 5' end	4q12

5.3. Identification of Candidate Gene

All the 13 candidates identified from MS-AP PCR showed homology to known or novel human mRNAs sequences, 10 of them were mapped to the 5' regions of known or novel genes (Table 5.4).

We first selected candidates with CpG island(s) mapped to 5' region of a known or novel gene (N=10), and finally we extend our target to candidates with CpG island mapped to coding region (N=1) and 3' region (N=2). The mRNA expression of these 13 known or novel genes in 5 ALL cell lines (697, CCRF-CEM, MOLT3, REH and Rs4;11) were examined by semi-quantitative RT-PCR. Among all, differential expression of *fibrillin 2* (*FBN2*) mRNA was found in the ALL cell lines (N=5) compared with normal PBMC (N=6) (will be discussed in sections 5.4), while the others (*FAT*, *PAX6*, *c14orf58*, *MLL*, *APS*, *ATXN2*, *ZHX2*, *PPFIA2*, *ZBED4*, *SRPX2*, *RNABP1* and *PDGFR-*α) showed no significant changes in mRNA expression between the ALL cell lines and normal PBMC. Hence, *FBN2* was selected for further studies.

Table 5. 4 *Thirteen putative target sequences those were adjacent to or within a defined CpG island.*

Seq No.	Differential methylation status	Corresponding CpG island	Corresponding Known mRNA	Gene name	Differential mRNA expression
1	Hypermethylation	3'	Tumor suppressor homolog 1 (Drosophila)	*FAT*	×
5	Hypermethylation	coding region	Paired box protein 6	*PAX6*	×
6	Hypomethylation	5'	Chromosome 14 open reading frame 58	*c14orf58*	×
8	Hypomethylation	5'	Myeloid/lymphoid or mixed-lineage leukemia	*MLL*	×
13	Hypermethylation	5'	SH2 and PH domain-containing adapter protein APS	*APS*	×
18	Hypermethylation	5'	Ataxin 2	*ATXN2*	×
19	Hypermethylation	5'	Fibrillin 2 precursor	*FBN2*	✓
23	Hypomethylation	5'	Zinc fingers and homeoboxes protein 2	*ZHX2*	×
24	Hypermethylation	5'	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2	*PPFIA2*	×
27	Hypomethylation	3'	Zinc finger, BED domain containing 4	*ZBED4*	×
28	Hypomethylation	5'	Sushi-repeat-containing protein, X-linked	*SRPX2*	×
33	Hypermethylation	5'	RAN binding protein 1	*RNABP1*	×
40	Hypermethylation	5'	Platelet-derived growth factor receptor, alpha polypeptide	*PDGFR-α*	×

5.4. *Fibrillin 2*

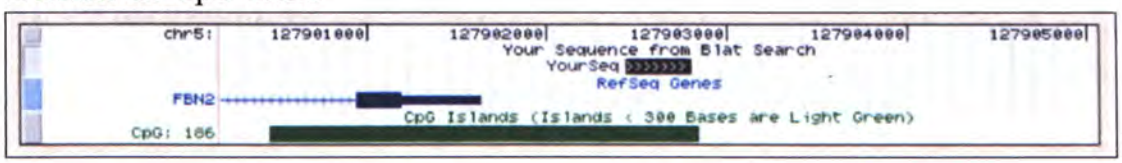
5.4.1. FBN2 CpG Islands: UCSC BLAT Search Analysis

UCSC BLAT analysis indicated that a CpG island was localized in the putative

promoter region of the *FBN2* (Figure 5.3). This CpG island sizes about 2382 bp in size and has a GC content of 59 % and an observed / expected CpG ratio of 0.90.

Figure 5. 3 *UCSC BLAT analysis of FBN2.*

As shown in the graph, GC percentage indicates the content of CG dinucleotides. *Black* rectangle indicates the location of the differentially methylated sequence identified. *Blue* rectangle indicates the location of the *FBN2* gene, and *green* rectangle indicates the CpG island.



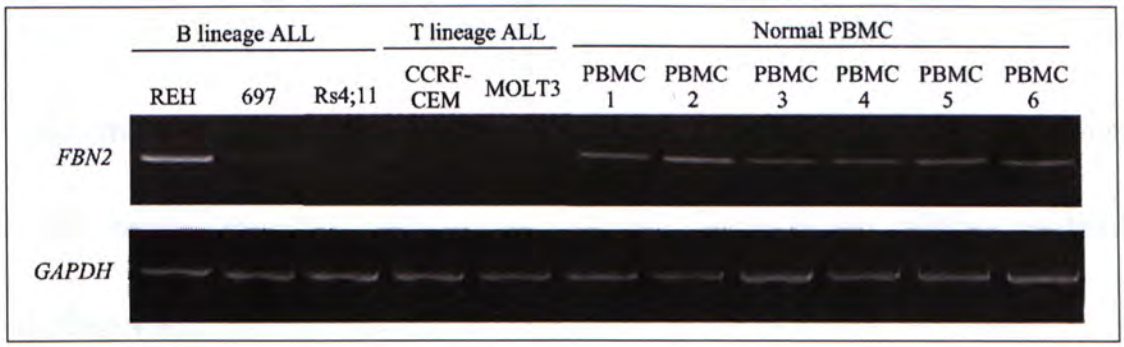
5.4.2. Verification of FBN2 by ALL Cell Lines

5.4.2.1. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR analysis of *FBN2* mRNA expression in 5 ALL cell lines and 6 normal PBMC samples were shown in Figure 5.4. Loss of expression of *FBN2* mRNA was detected in Rs4;11, CCRF-CEM and MOLT3 while reduced expression was observed in 697. In contrast, *FBN2* mRNA showed comparable level in REH as compared with the normal PBMC samples.

Figure 5. 4 *Semi-quantitative RT-PCR of FBN2 mRNA expression in 5 ALL cell lines and 6 normal PBMC samples.*

FBN2 mRNA expression was silenced in Rs4;11, CCRF-CEM and MOLT3 but down-regulated in 697. Amplification of *GAPDH* cDNA was performed as an internal control for RNA integrity.



5.4.2.2. COBRA

COBRA was performed to evaluate the methylation status of *FBN2* promoter in 5 ALL cell lines, 10 BMMC samples from non-malignant subjects and 10 normal PBMC samples from normal healthy donors. The PCR product was designed to span from nucleotide -479 to -76 of the *FBN2* putative promoter region. This region contained 39 CG dinucleotides and 3 *BstUI* restriction sites (Figure 5.5). All the non-malignant BMMC and normal PBMC samples were unmethylated as no methylated bands were observed (Figure 5.6B). As shown in Figure 5.6A, 2 methylated bands were detected in REH while 3 methylated bands were detected in Rs4;11, 697, CCRF-CEM and MOLT3, this indicated *FBN2* promoter hypermethylation occurred at different *BstUI* sites in all the 5 ALL cell lines (Figure

5.5A). An unmethylated band was observed in REH and we defined REH had a partial methylation (PM) while the other 4 ALL cell lines had no unmethylated band and their methylation status were defined as full methylation (FM). Demonstrated by COBRA, the relative methylation density in REH cell line was comparatively lower than other 4 ALL cell lines. This result was concordant with the mRNA expression results because the *FBN2* mRNA expression was found to be loss or down-regulated in those 4 ALL cell lines with higher methylation density as defined by COBRA.

Figure 5. 5 *A schematic diagram showing the primer design of COBRA and the position of BstUI cut sites in the FBN2 putative promoter region.*

The location of CG dinucleotides (*vertical red lines*) is indicated within the CpG island. The first nucleotide of exon 1 is marked with a *bent arrow* and assigned as +1. The region analyzed by COBRA and bisulfite sequencing spans from -479 to -76. Cleavage of the PCR product (404 bp) with *BstUI* yielded various bands (249 bp, 107 bp, 36 bp and 12 bp)

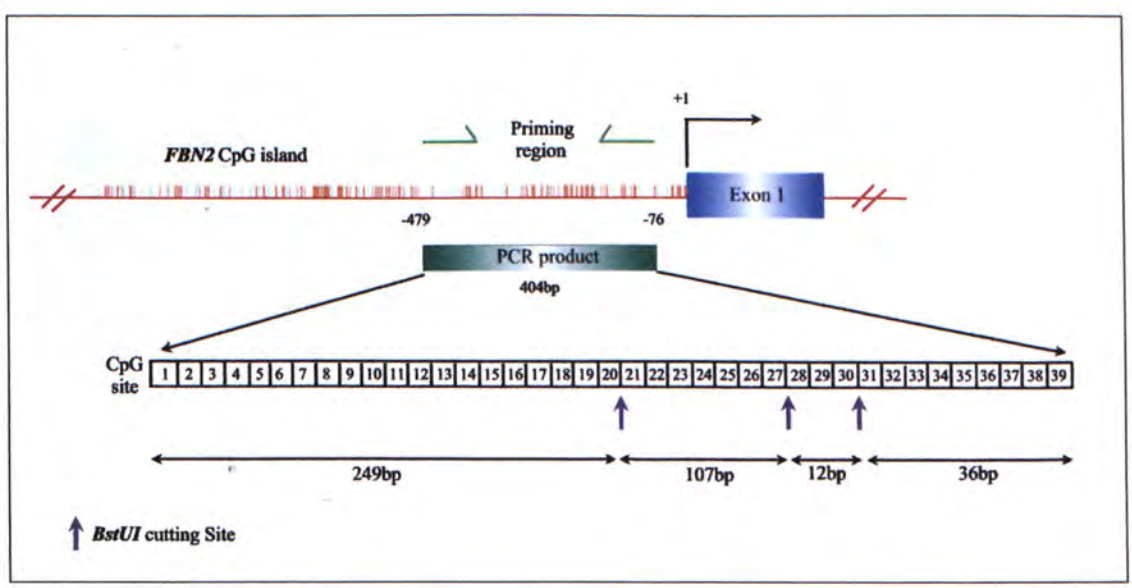
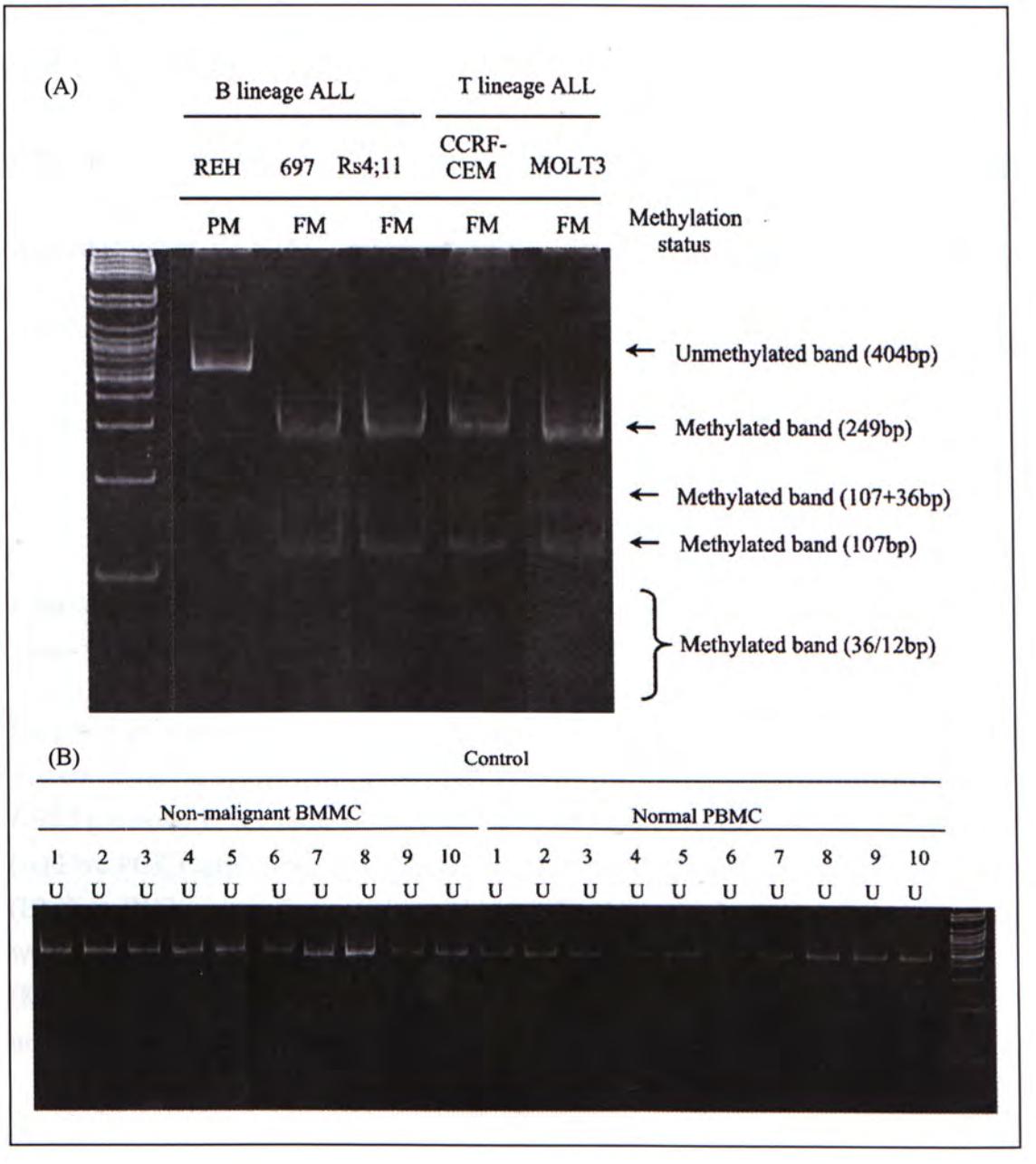


Figure 5. 6 *COBRA analysis of the FBN2 putative promoter region in 5 ALL cell lines.*

This gel picture shows the methylation status of the FBN2 promoter region in 5 ALL cell lines, 10 BMMC non-malignant and 10 normal PBMC samples. Unmethylated and methylated bands were indicated by arrows. The methylation status of each sample was indicated in the figure.
(Key: *U*: unmethylated; *M*: methylated; *FM*: full methylation; *PM*: partial methylation; *U*: unmethylated).



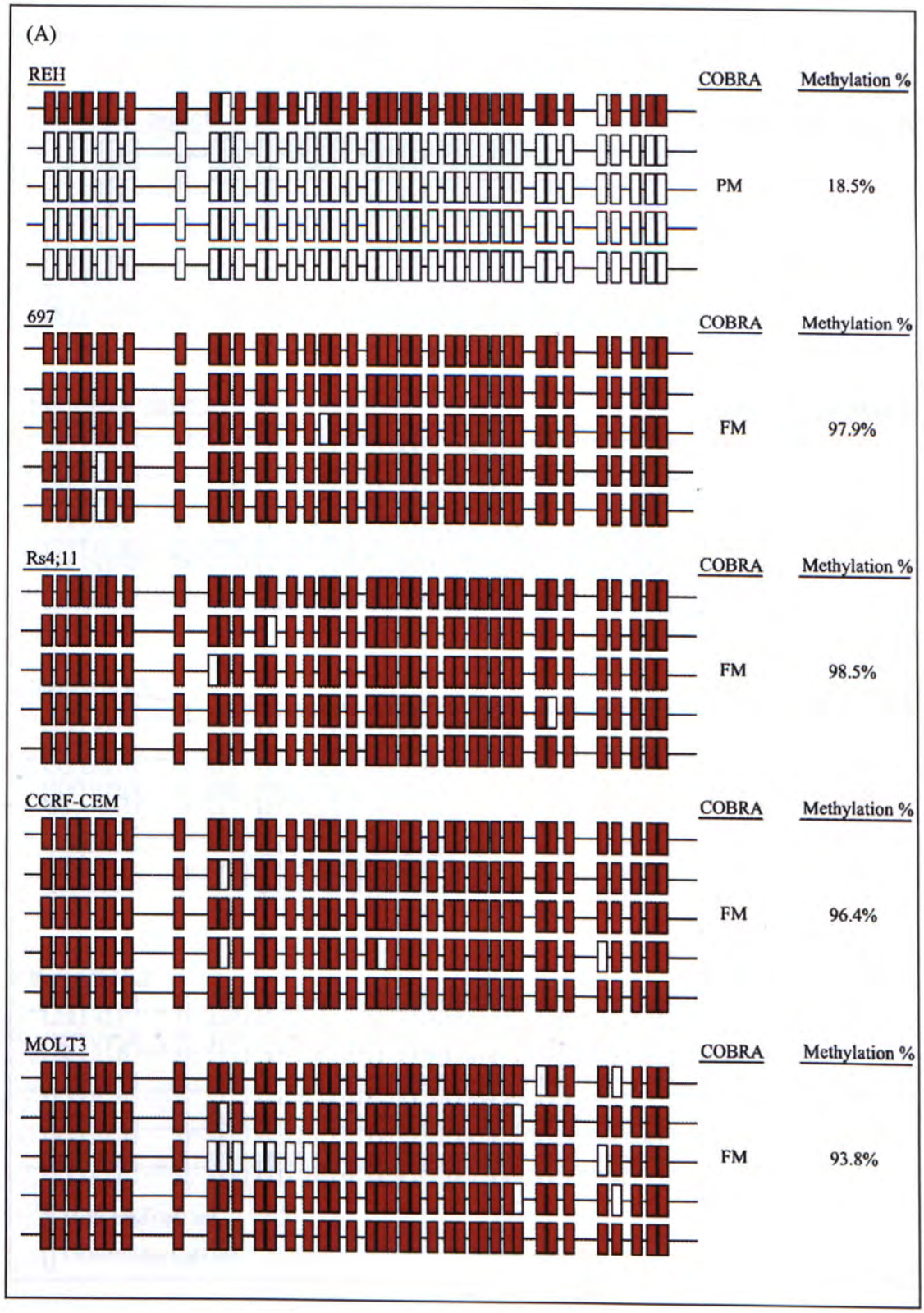
5.4.2.3. Cloned Bisulfite Sequencing

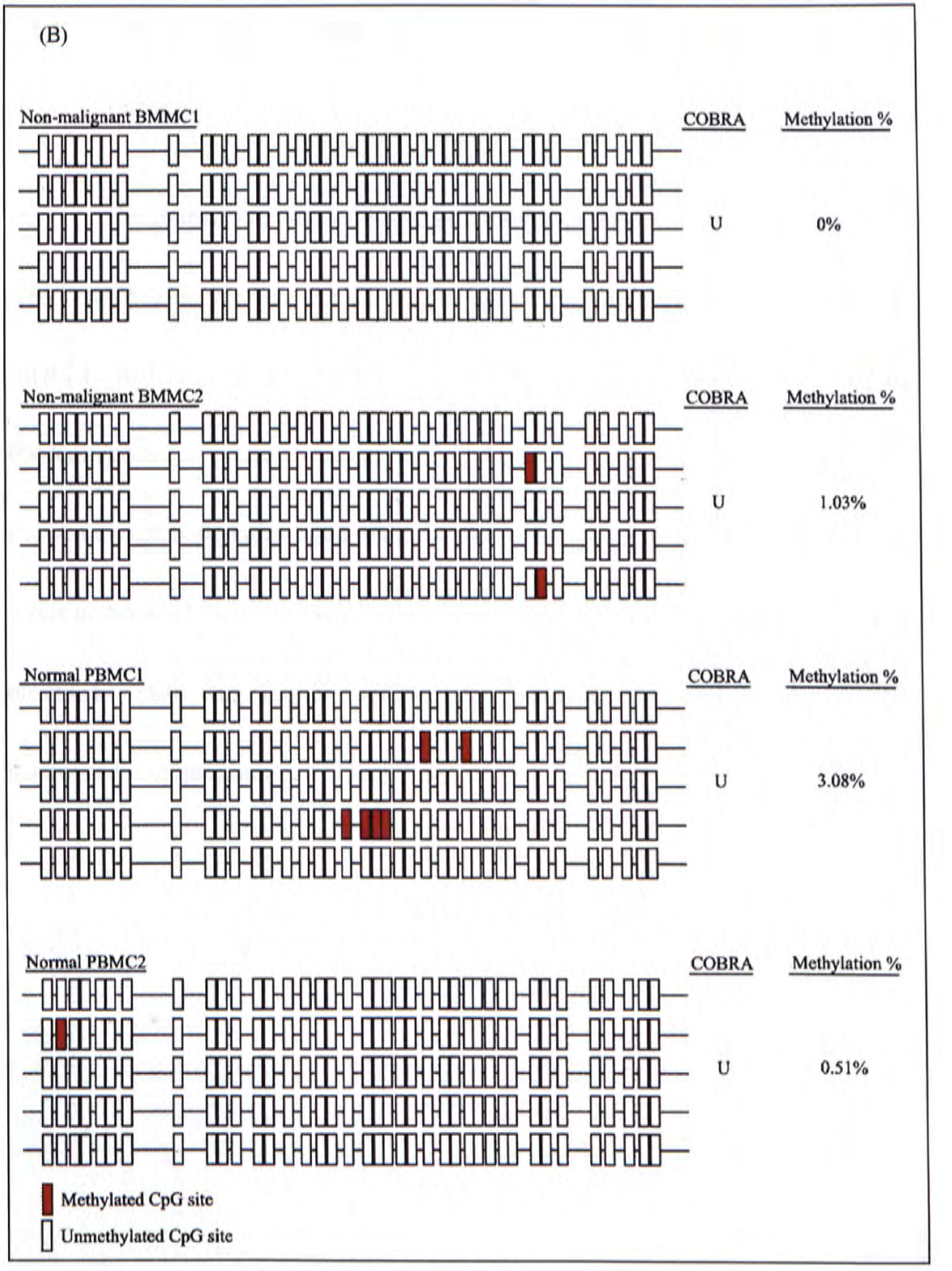
To determine the methylation density of *FBN2* putative promoter region, cloned

bisulfite sequencing was performed for 5 ALL cell lines, 2 BMMC samples from non-malignant subjects and 2 PBMC samples from normal healthy donors. Figure 5.7 showed that all the PCR clones analyzed were heavily methylated in the 4 FM cell lines, which had an overall methylation level of 97.9 % (697), 98.5 % (Rs4;11), 96.4% (CCRF-CEM) and 93.8 % (MOLT3) respectively. The methylation level of *FBN2* in REH was only 18.5 %. This low frequency of methylated CpG sites in REH was concordant with the semi-quantitative RT-PCR and COBRA results. Compare with our non-malignant BMMC and normal PBMC, the methylation frequencies were obviously lower than ALL cell lines.

Figure 5. 7 *Cloned bisulfite sequencing of FBN2 putative promoter in 5 ALL cell lines.*

Each row of squares represents 1 PCR clone, and each square represents a CpG site. The methylation status defined by COBRA and the overall methylation level of the *FBN2* promoter in each sample was shown in the right column.
(A) Five PCR clones from 5 ALL cell lines (REH, 697, Rs4;11, CCRF-CEM, MOLT3)
(B) Five PCR clones from 2 non-malignant BMMC and 2 normal PBMC samples were sequenced.
(Key: *red square*: methylated; *white square*: unmethylated; *M*: methylated; *U*: unmethylated)


(A)
REH
COBRA
Methylation %
PM
18.5%
697
COBRA
Methylation %
FM
97.9%
Rs4;11
COBRA
Methylation %
FM
98.5%
CCRF-CEM
COBRA
Methylation %
FM
96.4%
MOLT3
COBRA
Methylation %
FM
93.8%

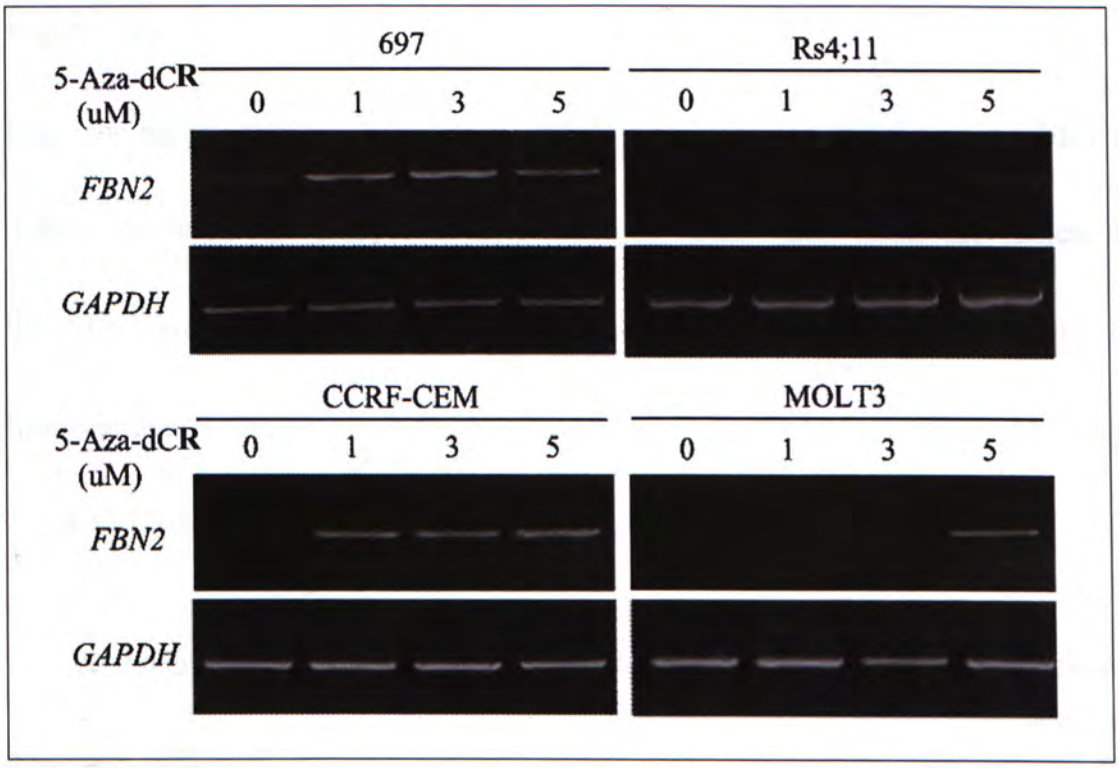

(B)
Non-malignant BMMC1
COBRA
Methylation %
U
0%
Non-malignant BMMC2
COBRA
Methylation %
U
1.03%
Normal PBMC1
COBRA
Methylation %
U
3.08%
Normal PBMC2
COBRA
Methylation %
U
0.51%
Methylated CpG site
Unmethylated CpG site

5.4.2.4. Demethylation Treatment Resorted FBN2 mRNA Expression in ALL Cell Lines

To confirm the role of *FBN2* promoter hypermethylation in the control of *FBN2* mRNA expression, *FBN2* mRNA non-expressing cell lines CCRF-CEM, MOLT3, Rs4;11 and down-regulated cell line 697 were treated with various concentrations of 5-aza-2'-deoxycytidine (5-Aza-dCR) for 96 hours. Figure 5.8 showed that demethylating agent induced a dose-dependent increase of *FBN2* mRNA levels in those cell lines. Conversely, no significant change in *GAPDH* expression was observed. These data further indicated that methylation of the *FBN2* promoter inactivated its expression in ALL cell lines.

Figure 5. 8 *Reactivation of FBN2 mRNA expression by 5-Aza-dCR.*

The non-expressing (CCRF-CEM, MOLT3, Rs4;11) or down-regulating (697) cell lines were treated with various concentrations (1, 3, 5 and 10 μM) of 5-Aza-dCR for 96 hours. Total RNA was extracted from untreated and treated cells for semi-quantitative RT-PCR analysis. *FBN2* mRNA expression was dose-dependently restored after 5-Aza-dCR treatment. Amplification of *GAPDH* cDNA was carried out as an internal control for RNA integrity.



5.4.3. Studies of FBN2 in Childhood ALL

5.4.3.1. Methylation Analysis

The contribution of *FBN2* promoter hypermethylation was validated in ALL cell lines by semi-quantitative RT-PCR, COBRA and cloned bisulfite sequencing. We next examined whether hypermethylation of the *FBN2* promoter could be detected in BMMC samples from pediatric patients with ALL. We used COBRA to evaluate

FBN2 promoter hypermethylation in 64 leukemic samples (Figure 5.9). The results showed that 69 % (44 out of 64) patients had *FBN2* promoter hypermethylation. The methylation frequency of ALL subtypes was summarized in Table 5.5. In addition, statistical analysis revealed *FBN2* promoter hypermethylation was common for both B and T lineage ALL (p=0.1649, >0.05, N=64 by Fisher's exact test). On the other hand, the methylation frequency in B lineage ALL subtypes showed no significant differences (p=0.307, >0.05, N=58 by Pearson chi-square test). These data indicated the *FBN2* promoter hypermethylation was not specific to cell lineages (B or T lineages) and B lineage subtypes (pro-B ALL, early pre-B ALL, common ALL and pre-B ALL) and thus is a common event in childhood ALL.

Promoter hypermethylation status provides important information for disease prognosis. However, statistical analysis on the *FBN2* methylation status showed no correlation with clinical risk groups as indicated by the chi-square test (p=0.092, >0.05, N=44).

Aberrant expression of myeloid markers CD13 and CD33 were observed in 42 among 64 patients in our clinical samples. We next examined the correlation between methylation status of *FBN2* and the aberrant expression of myeloid markers. The chi-square test result was significant (p=0.043, <0.05, N=38, by t-test) and we can

conclude that there is significant difference in the means of CD13 expression between promoter hypermethylation of *FBN2* patient at 5 % level of significance.

In order to validate *FBN2* methylation status with the progression of childhood ALL (further discussion can be found in section 6.5.3), statistical analysis was done to examine the correlation between *FBN2* methylation status with the white blood cell count (WBC) (N=59) and PB blast (N=59). However, no correlation was found between them.

To further evaluate the methylation status of each CpG sites, cloned bisulfite sequencing was performed on 4 BMMC from pediatric patients with ALL. Our data showed that varying degrees of methylation (29% to 87 %) were detected in these patients with *FBN2* promoter hypermethylation. In addition, all the clones from the two patients with no *FBN2* promoter hypermethylation showed clear unmethylation patterns (Figure 5.10). These data confirmed the presence of *FBN2* promoter hypermethylation in pediatric patients with ALL.

Table 5. 5 *Methylation frequency of childhood ALL lineages and subtypes.*

Types of Childhood ALL	Methylation frequency (%)
ALL	69 % (44/64)
B lineage ALL	66 %(38/58)
Pro-B ALL	100 % (1/1)
Early Pre-B ALL	100 % (5/5)
Common ALL	63 % (22/35)
Pre-B ALL	59 %(10/17)
T lineage ALL	100 %(6/6)

Figure 5. 9 *A representative gel image of COBRA of FBN2 putative promoter in childhood ALL.*

(A) COBRA of 8 BMMC samples from pediatric patients with B lineage ALL.
(B) COBRA of 6 BMMC samples from pediatric patients with T lineage ALL samples.
(Key: M: methylated; *U:* unmethylated)

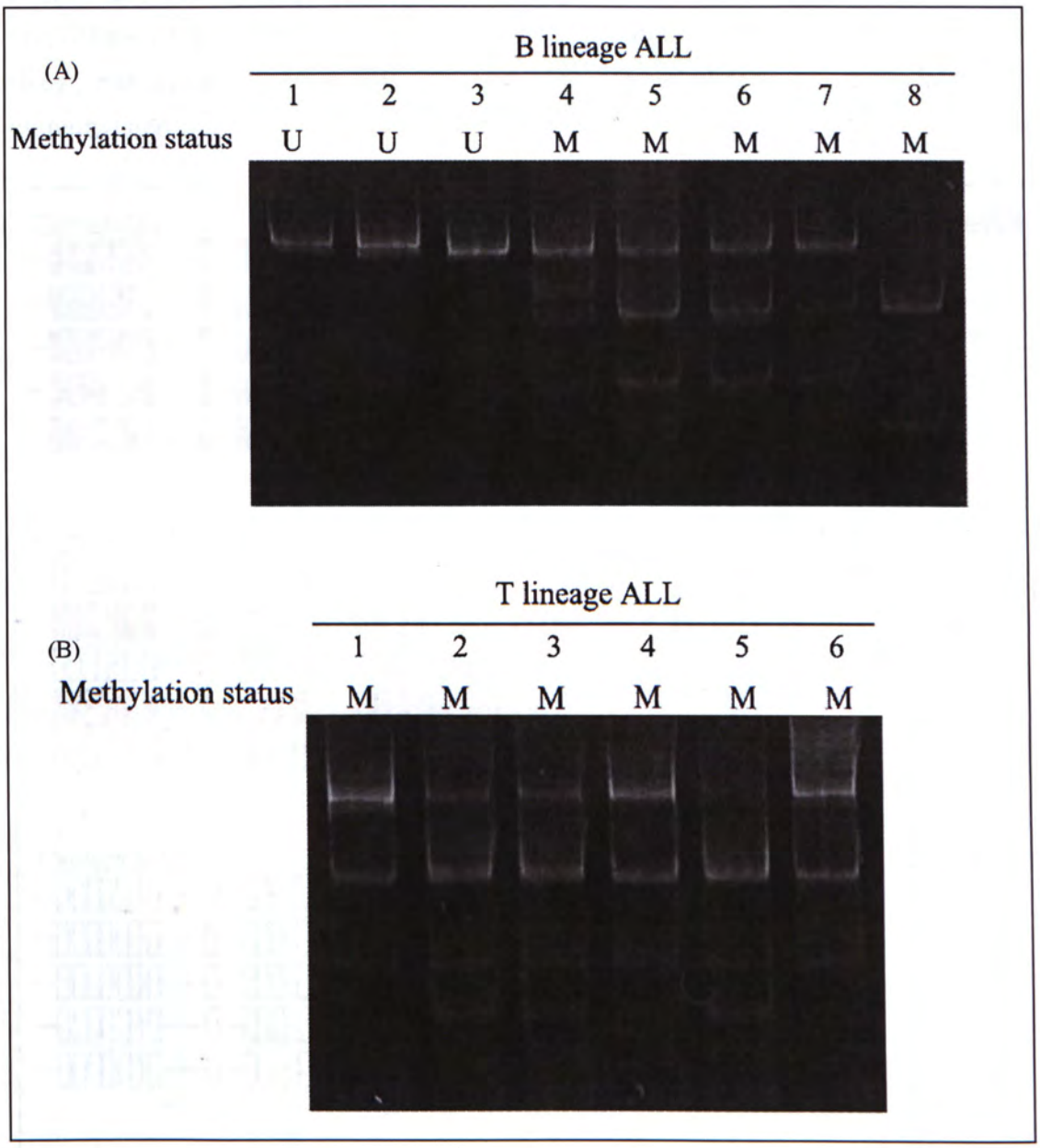
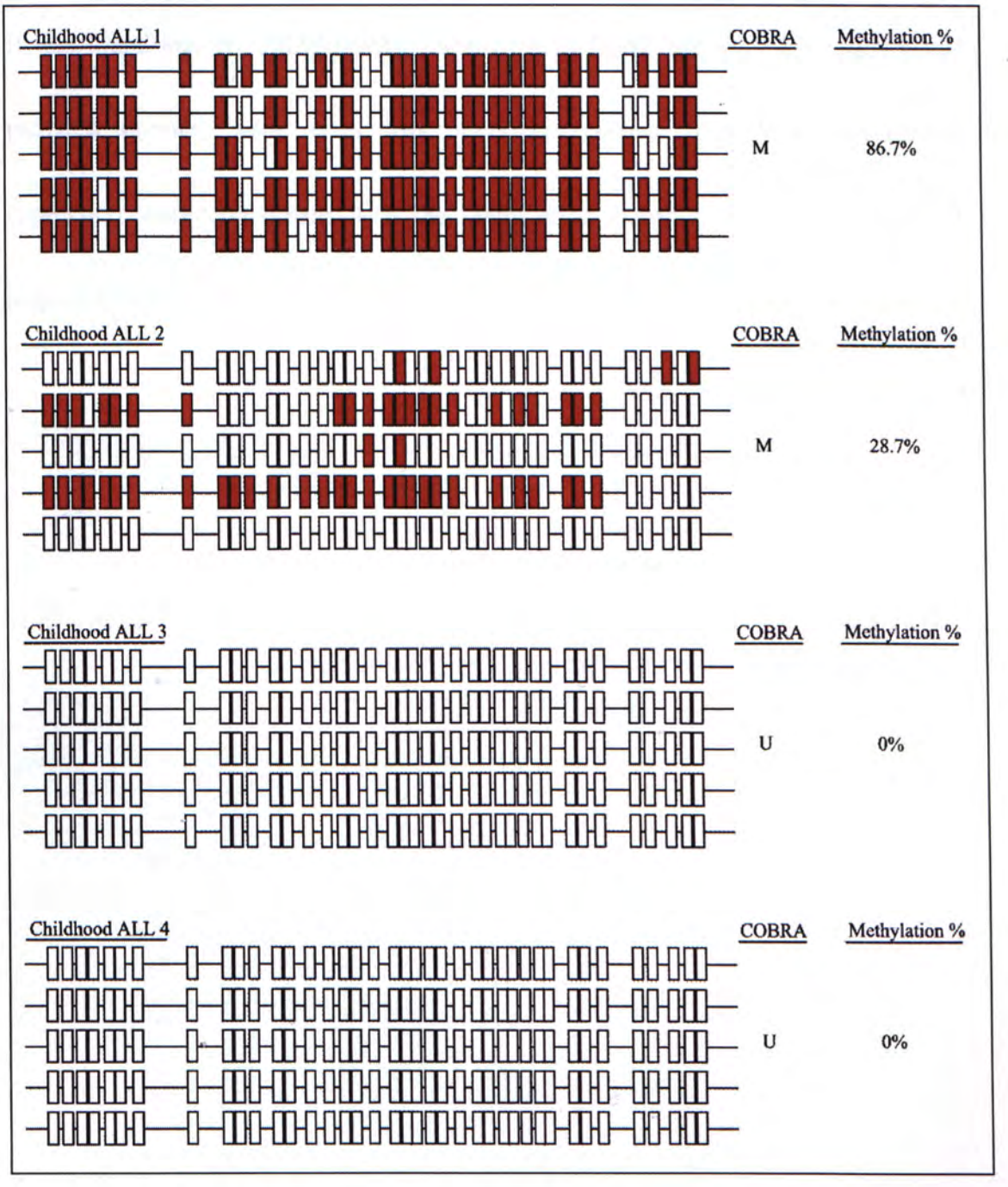


Figure 5. 10 *Cloned bisulfite sequencing analysis of the FBN2 putative promoter in pediatric patients with ALL.*

Five PCR clones from 4 BMMC samples from pediatric patients with ALL were sequenced. Each row of squares represents one PCR clone, and each square represents a CpG site. The methylation status defined by COBRA and the overall methylation level of the *FBN2* promoter in each sample was shown in the right column.
(Key: *red square*: methylated; *white square*: unmethylated; *M*: methylated; *U*: unmethylated)

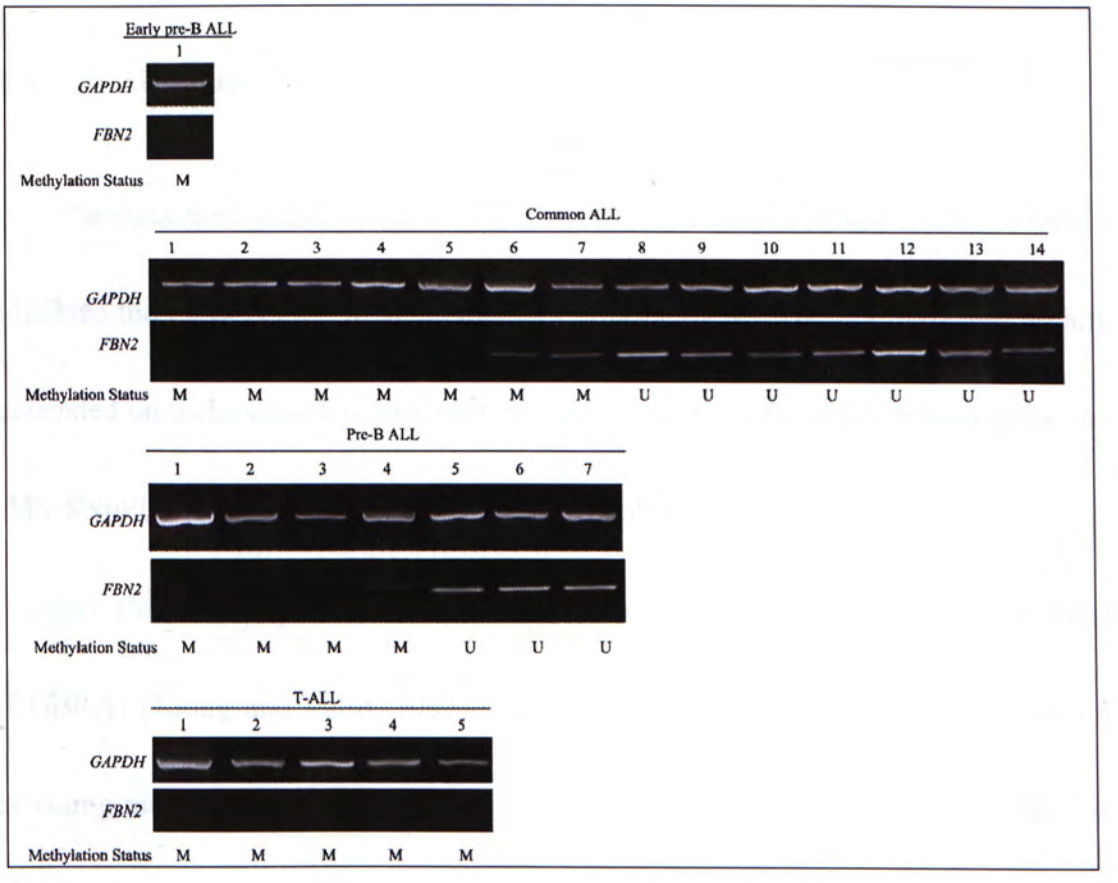


5.4.3.2. Semi-quantitative RT-PCR

After confirming the presence of *FBN2* promoter hypermethylation in childhood ALL, *FBN2* mRNA expression analysis was performed on 27 BMMC samples from pediatric patients with ALL using semi-quantitative RT-PCR. Among the samples, 1 was early pre-B ALL, 14 were common ALL, 7 were pre-B ALL and 5 were T-ALL. It was found that the *FBN2* mRNA expression in *FBN2 promoter* hypermethylated patient's samples were either loss or down-regulated. The results of mRNA expression were concordant with the methylation status as defined by COBRA (Figure 5.11).

Figure 5. 11 *Semi-quantitative RT-PCR analysis of FBN2 mRNA expression in 27 BMMC samples from pediatric patients with ALL.*

(Key: *M*: methylated; *U*: unmethylated)



Chapter 6 Discussion

6.1. Genome-wide Screening Approach: MS-AP PCR

Previous methylation studies in childhood acute lymphoblastic leukemia (ALL) involved the investigation of single-gene or multiple-gene, whose approaches usually depended on techniques like methylation-sensitive single nucleotide primer extension (MS-SNuPE) (Gonzalgo and Jones, 1997), methylation-sensitive polymerase chain reaction (MS-PCR) (Rust *et al.*, 1993) or combined bisulfite restriction analysis (COBRA) (Xiong and Laird, 1997). Although these techniques are widely used for detecting methylation changes at a specific locus, results are often limited to known methylation-regulated genes but not novel ones.

In response to various findings about methylation-induced silencing of tumor suppressor genes, genome-wide screening techniques for identifying DNA methylation pattern were developed in late 1990's (Ushijima *et al.*, 1997; Huang *et al.*, 1999). Therefore, we performed genome-wide DNA methylation screening using methylation-sensitive arbitrarily primed polymerase chain reaction (MS-AP PCR) to reveal novel cancer-related genes in childhood ALL regulated by CpG island methylation. Other genome-wide scanning methodologies include restriction

landmark genomic scanning (RLGS) (Kawai *et al.*, 1993), methylation-sensitive representational difference analysis (MS-RDA) (Ushijima *et al.*, 1997), methylated CpG island amplification coupled with representational difference analysis (MCA-RDA) (Toyota *et al.*, 1999), demethylating agent cDNA microarray (Suzuki *et al.*, 2002; Hatada *et al.*, 2006) and differential methylation hybridization (DMH) (Huang *et al.*, 1999). In particular, these methods allow the screening of more than thousands of methylated sequences. For examples, RLGS can screen more than 2000 DNA sequences, whereas MS-RDA and MCA-RDA are able to screen up to 10^4-10^5 DNA sequences. Nevertheless, these methods are technically complicated which require a large amount of starting material (DNA and RNA) and as a result, might suffer from interference by repetitive sequences (Ushijima, 2005).

Conversely, we selected MS-AP PCR because of its simplicity and reproducibility. For instance, this screening approach has been employed to identify novel hypermethylated genes such as *transmembrane protein with EGF-like and two follistatin-like domains 2* (*TMEFF2*), *paired box gene 6* (*PAX6*), *pituitary tumor apoptosis gene* (*PTAG*) and *LIM homeobox 6 gene* (*LHX6*) in colon cancer, bladder cancer, pituitary adenomas and head and neck squamous cell carcinoma respectively (Liang *et al.*, 2000; Salem *et al.*, 2000; Bahar *et al.*, 2004; Estecio *et al.*, 2006). Besides, another similar DNA fingerprinting technique called MS-RF has been used

to identify *peroxisomal membrane protein 24kDa* (*PMP24*) and *endothelin receptor B* (*EDNRB*), which are aberrantly hypermethylated in prostate and nasopharyngeal carcinomas (Davies, 2002, Lo *et al.,* 2002; Wu and Ho, 2004). These findings solidified the use of MS-AP PCR as a feasible system for genome-wide methylation screening.

6.2. Sample Selection in this Study

6.2.1. MS-AP PCR

Sample preparation and selection are the early steps of genome-wide screening. To identify methylation-regulated genes in childhood ALL by MS-AP PCR, methylation patterns between leukemic and control samples were compared. Moreover, the selection of clinical samples of pediatric patients with ALL was based on leukemic blast count and only patients having leukemic blast count ≥90 % were used (blast count ≥30 % is destined to the diagnosis of ALL). Therefore, underestimation of methylation interfered by normal cells can be minimized.

6.2.2. Methylation Studies

To prepare clinical samples for methylation studies, it is important to have a homogeneous population of cells. BM samples from pediatric patients with ALL may contain normal heterogeneous hematopoietic cells and for this reason, the degree of methylation may be underestimated. For instance, recent studies revealed there are cell-type specific DNA methylations (Futscher *et al.,* 2002; Ohgane *et al.,* 2002; Ohgane *et al.,* 2005). To overcome this drawback, we isolated MC (mainly composed of leukemic blasts) from BM of pediatric patients with ALL using Ficoll-Paque™ PLUS gradient centrifugation. It is more appropriate to select corresponding normal BM as control in the methylation analysis. However, normal BM samples are very limited. To tackle this problem, DNA from non-malignant BMMC and normal PBMC were used as they are generally accepted as a normal control for ALL methylation study (Wong *et al.*, 2000; Agirre *et al.*, 2006).

6.2.3. Studies in ALL Cell Lines

In order to achieve a preliminary result on the mRNA expression and promoter hypermethylation of putative candidate genes, semi-quantitative RT-PCR and COBRA were performed in selected ALL cell lines, to be benefited from the unlimited supply of DNA from cell culturing and homogenous nature of cells. Therefore, ALL cell lines

originated from B and T lineages were use for first-line screening prior to clinical sample analyses.

6.3. Methylation Patterns in Childhood ALL

Our present study provided the first estimation of genome-wide methylation changes in childhood ALL. The MS-AP PCR scans the CG-rich regions in the human genome and provides information about the methylation changes in individual samples.

Our MS-AP PCR results showed that there was no significant difference of methylation frequencies between leukemic group and control group as indicated in Table 5.1. This insignificance might be due to the small sample sizes in our studies. Conversely, studies in the colon cancer demonstrated a significant reduction in 5-methycytosine level in adenomas and adenocarcinomas when compared with normal and paired colonic mucosa (Feinberg *et al.*, 1988). Hence, changes in methylation frequency should not be excluded in childhood ALL.

6.4. Candidate Genes Selection Strategies in MS-AP PCR

By using MS-AP PCR, we initially identified 40 evaluable sequences exhibiting potential differential methylation changes in childhood ALL (Table 5.1). We then analyzed their genomic localization by UCSC BLAT analysis and we found that 25 of 40 (63 %) sequences were mapped to a known or novel gene (Table 5.3). We further selected those sequences that are corresponded to any known or novel mRNA. Among the short-listed ones, 13 were identified as transcript-associated CpG islands (located at 5' region, coding region or 3' region of a gene) and 10 of them were mapped to 5' region of a gene. These 13 sequences were mapped to known genes which have not been reported to associate with methylation-induced tumorigenesis in ALL (Figure 5.4). Recent studies revealed methylation of transcribed regions and regions near regulatory elements also involve in the gene expression (Aranyi *et al.*, 2005; Meng *et al.*, 2003). Therefore, we did not limit the selection to 5' CpG islands and isolated transcript-associated CpG islands during validation.

Semi-quantitative RT-PCR was performed to examine the mRNA expression level of these 13 putative candidate genes. However, 12 of the putative candidates did not show a significant change in expression levels in the ALL cell lines. Finally, we identified a potential candidate gene *fibrillin 2* (*FBN2*). The *FBN2* showed

down-regulation of mRNA expression in 4 out of 5 ALL cell lines (697, CCRF-CEM; MOLT3, Rs4;11) and was thus subjected to further studies.

6.5. Fibrillin 2: mRNA Expression and Methylation Studies

6.5.1 ALL Cell Lines

Our results demonstrated the aberrant *FBN2* promoter hypermethylation was concordant to its loss of (CCRF-CEM; MOLT3, Rs4;11) or down-regulated (697) mRNA expression in 5 ALL cell lines. Although promoter hypermethylation was detected by COBRA in REH, cloned bisulfite sequencing revealed not all the clones from REH were methylated; in other words, low methylation density in REH didn't affect the gene expression of *FBN2*. Demethylating treatment performed by 5-Aza-dCR on *FBN2* methylated and non-expressing cell lines reactivated their *FBN2* mRNA expression, suggesting that the gene silencing in these cell lines were mediated by methylation. All these results suggested *FBN2* promoter hypermethylation does play an important role in childhood ALL.

6.5.2 Childhood ALL

6.5.2.1 mRNA Expression and Methylation Studies

Our data indicated the loss of *FBN2* mRNA expression by promoter hypermethylation was a common event in childhood ALL. It was found that the *FBN2* mRNA expression in *FBN2 promoter* hypermethylated patient's samples were either lost or down-regulated (Figure 5.11).

Scattered methylation of CpG sites were found in some of the non-malignant BMMC and normal PBMC control samples by cloned bisulfite sequencing, which contributed to 0.51 %, 1.03 % and 3.8 % respectively (Figure 5.7B). The methylation frequencies of these controls are much lower than those heavily methylated ALL cell lines (Figure 5.7A). In addition, the *FBN2* mRNA expression seems not to become inactivated under low methylation frequency in such control samples (Figure 5.6B). The detection of methylated CpG sites in control samples can be explained by 2 easons. First, our normal controls were recruited from adult subjects. It is because the recruitment of non-malignant BMMC and normal PBMC from pediatric subjects are very limited. Hence, all the controls in this study were recruited from non-malignant or normal healthy adults. Therefore, detection of methylated alleles in the controls might represent pre-malignant changes or be related to age (Tra *et al.*, 2004). Second, the low percentage of methylation found in normal controls may be arisen from random events and did not affect gene expression. It was found that scattered methylation of CpG sites is common in cancer control samples but these did not

silence the gene expression provided that they are methylated at low frequencies (Liu *et al.*, 2006).

6.5.2.2 Statistical Analysis

In order to understand the correlation between methylation statuses of *FBN2* between clinical risk groups, we performed statistical analysis to reveal their correlation. It is because there is a lack of relevant reports discussing these issues and most of the current studies failed to demonstrate a strong correlation between methylation status and clinical outcomes of ALL. However, no significance findings were found with clinical risk groups, which may probably due to the small sample size in our study.

On the other hand, the aberrant expression of myeloid marker CD13 was found to be statistically significant with hypermethylation status of *FBN2* (p=0.043, <0.05 by chi-square test). To our knowledge, it is the first study demonstrating such relationship between methylation status and aberrant expression of myeloid marker in hematological malignancies. However, the contribution of aberrant expression of myeloid marker in childhood ALL is still unknown although it is regarded as an aberrant phenotype in childhood ALL. It was found that Philadelphia chromosome-positive ALL patients have a poor prognosis and they are usually

associated with higher incidence of aberrant myeloid markers expression (Uckun *et al.*, 1998; Boldt *et al.*, 1994). On the contrary, the direct correlation between aberrant expression of myeloid markers and disease prognosis of ALL has not been discussed in the literatures. The relationship between *FBN2* promoter hypermethylation, aberrant expression of myeloid marker and patient prognosis needs further investigation to confirm the hypothesis.

6.5.3. Possible Roles of FBN2 in Leukemogenesis

*FBN2 p*romoter hypermethylation has been demonstrated in non-small cell lung cancer (NSCLC) (49 %, 62/126) and pancreatic cancer (75 %, 18/24) (Chen *et al.*, 2005; Hagihara *et al.*, 2004). *FBN2* promoter hypermethylation induced down-regulation of *FBN2* mRNA expression was also observed in NSCLC primary tumor and pancreatic cell lines. Reactivation of *FBN2* mRNA expression by demethylating agent was demonstrated in all *FNB2* mRNA non-expressing NSCLC cell lines (Chen *et al.*, 2005). These studies suggested that promoter hypermethylation and mRNA expression silencing of *FBN2* in tumor cells may play important roles in tumorigenesis.

FBN2 gene is located at chromosomal region 5q23.1. It is a large modular

extracellular matrix (ECM) glycoprotein and is a key component of ECM. Defect of *FBN2* is genetically linked to congenital contractural arachnodactyly, a connective tissue disorder (Putnam *et al.*, 1995). Since little is known about the role of FBN2 protein in tumorigenesis or leukemogenesis, we performed literature review to characterize the functional domains of FBN2 protein.

FBN2 protein has 4 well-characterized motifs or domains: RGD peptide, transforming growth factor (TGF)-β-binding protein-like domain, epidermal growth factor (EGF)-like domain and the calcium binding EGF domain (Sakamoto *et al.*, 1996; Pereira *et al.*, 1993; Zhang *et al.*, 1994). RGD sequence is an arginyl-glycyl-aspartyl peptide and is most likely being recognized by the beta1 receptor by serving as a ligand for alpha 3, alpha (v) and beta1 integrin receptor. (Sakamoto *et al.*, 1996; D'Arrigo, 1998). FBN2 protein was found to be biologically active for cell adhesion (Ritty *et al.*, 2003) and beta1 integrin does play an important role in the interaction between hematopoietic cell and ECM proteins. The involvement of TGF-β signaling pathway in hematopoiesis is still controversial. However, TGF-β signaling pathway has an important role in solid tumor like colon, pancreatic breast and prostate cancer. Nevertheless, the disruption of TGF-β signaling pathway alone doesn't affect hematopoiesis. Therefore, it may be involved in progression rather than the initiation of leukemia (Lin *et al.*, 2005). Besides, we didn't

find any direct evidence on leukemogenic involvements of EGF-like domain and calcium binding-EGF domain of FBN2 protein.

ALL is a clonal hematopoietic disorder by cell maturation arrest and accumulation of malignant lymphoblasts in marrow, lymphatic and non-lymphatic tissues and in most cases, leukemic blasts migrate from the BM into PB (Geijtenbeek *et al.*, 1999). Therefore, aberrant ALL progenitor proliferation, differentiation and homing could be correlated to altered adhesion properties of the ALL blasts. Based on the literatures mentioned, the role of FBN2 protein as a beta1 integrin ligand seems to play a particular role in leukemogenesis (Sakamoto *et al.*, 1996; D'Arrigo, 1998). Among the adhesion molecules, the integrins of beta1 family are known to direct cell-cell and cell-matrix interaction (Liesvel *et al.*, 1993). Evidence has been provided that CD 34 positive stem cells bind either to the bone marrow stroma or the ECM protein through the beta1 integrins (Simmons *et al.*, 1992; Hynes, 1992). Even though the exact mechanisms of adhesion of ALL blasts to BM stroma are still unclear, beta1 integrin reorganization sites in FBN2 have been recognized to mediate those cellular interactions that are important in ALL biology. Based on this hypothesis, we perform statistical analysis to correlate the FBN2 methylation status with the WBC and PB blast. However, from the data we have obtained, the leukemogenic role of FBN2 seems not related to tumor invasion and metastasis as we expected. The leukemogenic

role of FBN2 affect pathway other than tumor invasion and metastasis. Thus, further study is required to understand the role of *FBN2* in the pathogenesis of childhood ALL. To study the role of FBN2, we can simply perform cDNA micorarry analysis on FBN2 knock-down ALL cell lines. Target leukemogenic pathway affected by FBN2 can be identified by comparing the gene expression among the *FBN2* positive and *FBN2* negative cell lines.

6.6. Clinical Application of *FBN2* Aberrant Methylation

6.6.1. Tumor Markers

The identification of differentially methylated patterns has potential clinical significance in being a tumor marker. For example, the methylation differences between tumor and normal cells can be used to detect the presence of tumor cells in biopsy specimens or to identify tumor-derived DNA in blood samples (Esteller *et al.*, 1999; Wong *et al.*, 1999; Kawakami *et al.*, 2000). Cancer specific methylation detection has been described in sputum biopsy for lung cancer and pancreatic juice for pancreatic cancer (Laird, 2003; Belinsky, 2004; Matsubayashi *et al.*, 2006). In this application, methylation is not necessary to induce gene silencing but is required to be specific to tumor cells or to have a correlation with clinical outcome of the patients. So far, no methylation-specific tumor markers have been found in childhood ALL. In

the succeeding year, this application has an increasing importance even the methylation does not induce gene silencing.

Methylation of specific genes or methylation profiling can also be linked to disease prognosis and response to chemotherapy. Promoter hypermethylation of *death-associated protein kinase 1* (*DAPK*) is associated to recurrent bladder cancer (Tada *et al.*, 2002). Besides, the inactivation of O^6-*Methylguanine DNA methyltransferase* (*MGMT*) through promoter hypermethylation leads to the increase of sensitivity of alkylating drugs used in chemotherapy in treating gliomas (Esteller *et al.*, 2000).

The criteria of *FBN2* in being a tumor marker have been demonstrated in our study, as implied from the high methylation frequency (69 %) observed in childhood ALL patients. Therefore, the promoter hypermethylation of *FBN2* can be regarded as a suggestive candidate as a tumor marker of childhood ALL. Due to the sensitivity of DNA molecular studies, the detection of remaining leukemic blasts or the possibilities of relapse, such as the occurrence of minimal residual disease (MRD) would become more precise. In particular, the down-regulation of FBN2 mRNA was found to be associated with its promoter hypermethylation. This may affect its protein expression and hence alters specific physiological pathway, on the other hand, affecting the prognosis of a patient. Hopefully, the role of *FBN2* can stride forward to act as a

prognostic marker when its functional characterization has demonstrated in ALL leukemogenesis.

6.6.2. Use of Demethylating Drugs in Chemotherapy

DNA-methyltransferases (DNMT) and histonedeacetylase (HDAC) are the 2 major drug targets for epigenetic inhibition. These provide a powerful rationale for using azanucleosides as a novel means of pharmacologic targeting of cancer cells that is distinct from low-dose chemotherapy. Decitabine (5-aza-2'-deoxycytidine), a cytosine analogue, has selective DNA demethylating activity at low doses (Momparler, 2005). A clinical development program with low-dose decitabine in malignant diseases is currently focused on myelodysplastic syndrome (MDS), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) (Issa *et al.*, 2004; Gore *et al.*, 2006). The optimal use of decitabine may be used in combination with HDAC inhibitors to promote gene expression. Optimized decitabine doses and combinations with other epigenetic therapies can be used at minimally toxic doses which provide potentially safer therapeutic options and introduce novel combination therapies (Gore, 2005).

Furthermore, the *in vivo* demethylating action has been demonstrated by our study using ALL cell lines as model. Hopefully, the application of epigenetic drugs

can be successfully applied to various cancers including childhood ALL.

6.7. Limitations of Methylation Studies

6.7.1. MS-AP PCR

Genome-wide methylation screening is a useful technique to identify difference in methylation patterns between cell types. However, it is important to emphasize its potential pitfalls. MS-AP PCR suffered from a few limitations. First, the numbers of bands analyzed on a single gel are limited. Second, only small sample size can be achieved and the number of samples per group is small. The small sample size does not allow sounding statistical analysis of the correlation between methylation changes and their clinical parameters to be performed. Therefore, increases in sample size would facilitate the analysis of the methylation changes. Lastly, MS-AP PCR has a relatively low resolution compared with other known techniques. However, we still utilize the MS-AP PCR platform because of the easy setup, simple technique and less labour work.

6.7.2. Techniques Used in Methylation Study

COBRA and bisulfite sequencing were performed in the candidate gene

methylation analysis. Though COBRA provides a simple and rapid scanning of methylation status, it may omit and underestimate methylation changes outside the restriction enzyme recognition sites. Therefore, more than one restriction enzyme can be included in COBRA to increase the number of CpG sites being detected. Moreover, this problem can be solved by cloned bisulfite sequencing, which however, it is labor intensive and more tedious, but it provides information on the methylation status of every cytosine residue within the target sequence. Hence, using both methods can facilitate a rapid and promising methylation analysis.

6.7.3. Problems in Methylation Study

It has been recognized that methylation in cancer cells frequently occurr in CpG islands outside promoter region which do not repress gene transcription. An example of this phenomenon is the hypermethylation of CpG island in exon 5 of *PAX6* in colon cancer (Salem *et al.*, 2000). Since not all CpG islands are localized to the 5' promoter region, methylation of these CpG islands usually may not regulate respective gene transcriptions. Moreover, promoter hypermethylation of a gene occuring in the promoter region may not be regarded as a tumor suppressor gene (Wutz *et al.,* 1997; Gonzalgo *et al.,* 1998). Even in normal cell, methylation of specific CpG islands

frequently occurs.

Genome-wide methylation screening usually results in isolation of numerous differentially methylated DNA fragments. To correlate the methylation status with a potential tumor suppressor gene, several verification steps are necessary. First, promoter hypermethylation associated with gene silencing must be identified. Second, the expression of a gene in normal cell must be confirmed. Third, the role of methylation in this particular gene can be verified by treating cells with demethylating agent. Finally, the functional role of the gene should be demonstrated in the participation of tumorigenesis. These made the identification of tumor suppressor gene by genome-wide scanning technique more complicated and time-consuming through a series of validation. Nevertheless, this problem is not restricted to MS-AP PCR. Other methylation screening approaches also identified the CpG-rich sequences outside 5' promoter region (Davies 2002; Liang *et al.*, 2002).

Although promoter hypermethylation has been demonstrated in various types of human cancers, hypomethylation is consistently observed (Hoffmann and Schulz, 2005). Hypomethylation mainly occurs in repetitive sequence, where high rates of mutation and polymorphism are observed. The analysis on quantifying hypomethylation is very difficult because large amount of DNA has to be used for this kind of repetitive sequence (Santourlidis *et al.*, 1999). In conclusion, the use of

hypomethylation as disease markers will therefore be very difficult.

6.8. Future Studies

In order to characterize the function of *FBN2* in childhood ALL pathogenesis, FBN2 protein expression should be done. It can be achieved by either Western-blotting, immuno-histochemistry or flow cytometry. Once the correlations between promoter hypermethylation and FBN2 protein expression have been verified, functional characterization of FBN2 protein can be performed to evaluate the role of *FBN2* in leukemogenesis. It can be done by electroporation of *FBN2* construct into FBN2 protein deficient ALL cell lines. The Nucleofection® (Amaxa Biosystems,Geithersburg, MD) is a newly developed technology for efficient non-viral transfection of suspension cell types. The transfection of recombinant plasmid into human CD34+ hematopoietic cell has been successfully demonstrated (Takeda *et al.*, 2006). The effect of re-expression of FBN2 can be monitored by cell cycle analysis, apoptosis assay and cell proliferation assay.

Moreover, to further confirm the possible significance of *FBN2* promoter hypermethylation in childhood ALL diagnosis or prognosis, a larger study with more detailed clinical outcomes of each patient is essential to understand the actual clinico-pathological role of *FBN2*. Once the prognostic value of *FBN2* is established,

individualized treatment can be applied on patients with *FBN2* promoter hypermethylation by epigenetic therapy or other specific chemotherapy.

Chapter 7 Conclusion

This is the first study investigating the genome-wide DNA methylation changes in childhood ALL patients. Our results demonstrated the feasibility of MS-AP PCR in the identification of methylation-regulated genes. For instance, we have successfully demonstrated the correlation between *FBN2* promoter hypermethylation with its mRNA down-regulation in various subtypes of childhood ALL.

FBN2 may be added to the list of methylation associated genes in childhood ALL as implied from the observable methylation frequency. On the other hand, these data suggested the use of *FBN2* promoter hypermethylation as a potential tumor marker for childhood ALL.

Nevertheless, the potential biological functions of FBN2 protein in human leukemogenesis remain to be elucidated. Functional characterization on the role of FBN2's biological functions may provide more information to understand the pathogenesis of childhood ALL.

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Appendix- Clinical Data of Samples Used in this Study

Case No.	Subtype	Methylation Status	Sex	Age	Blast count(%)	PB blast count (10^9/L)	PB Blast%	WBC Count (10^9/L)	Risk group	HKADR	CD10	CD19	CD20	CD22	CD2	CD3	CD3 cyto	CD5	CD7	TdT	CD13	CD14	CD33	MPO cyto	IgM	IgM cyto	CD16	CD34	CD79a cyto	CD15	CD68	Aberrant expression
1	common ALL	U	M	5	-	88.50	90%	98.30	-	96.3	91	95.3	2.9	91.9	2.9	-	-	3	3.5	-	2	0	2	-	-	negative	-	-	-	-	-	-
2	common ALL	M	F	15	-	5.80	57%	10.20	-	90.6	94.1	94.7	94.6	94.2	7.7	-	-	6.7	7.1	-	5.3	0.7	2.3	-	negative	-	-	-	-	-	-	-
3	common ALL	M	M	2	-	-	-	2.20	-	72.6	93.9	94.5	1.1	45.1	5.6	-	-	4.8	5.4	-	8.2	0.2	12.3	-	-	-	-	-	-	-	-	-
4	common ALL	M	M	8	95%	0.10	3%	2.10	-	87.9	94.5	94.3	36.3	93.8	7.1	-	-	7.5	9.2	-	11.3	1.5	19.2	-	-	0	-	-	-	-	-	-
5	common ALL	M	M	12	99%	0.01	29%	4.40	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	common ALL	M	F	1	93%	0.10	2%	4.80	SR	98	98.1	89.7	4.9	98.7	1.1	0.7	0.3	2.3	0.9	54.9	11	0.9	34.6	0.1	-	3.4	5.9	99.1	85	2.7		-
7	common ALL	M	F	4	96%	129.50	86%	150.60	IR	98.4	96	90.9	93.4	93.5	0.7	0.9	7	2.1	1.7	61.2	11.7	0.6	0.5	1.1	-	12.5	0.9	3.3	83.7	15.8		-
8	common ALL	M	M	3	64%	-	-	2.00	SR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	common ALL	M	F	3	95%	0.40	10%	3.80	-	45.2	96.2	96.9	20.7	92	1	-	-	1.9	2.1	-	0.5	0	0.1	-	-	-	-	-	-	-	-	-
10	common ALL	M	M	3	91%	6.10	77%	7.90	SR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	common ALL	M	M	14	50%	24.80	18%	138.00	HR	99.1	97.9	92.7	1.7	94.9	1.5	2.3	0.7	4.6	25.4	57.6	97.9	3.3	65.9	3.7	-	1.2	1.7	99.3	91	17.2		-
12	common ALL	U	M	3	80%	8.50	67%	12.70	SR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	common ALL	U	F	8	92%	23.70	78%	30.40	IR	99.9	98.7	96.8	8.7	92.4	1.4	0.5	8.4	1.4	0.9	49.6	4.6	2	13.8	0	-	2.9	2.1	52.5	86	16		-
14	common ALL	U	M	3	98%	26.43	59%	44.80	-	83.5	88.2	88.8	58	80.5	13.1	-	-	10.4	11.8	-	5.5	0	5.8	-	-	-	-	-	-	-	-	-
15	common ALL	U	F	5	80%	1.95	31%	6.30	-	79.2	84.6	88.7	3.6	84.9	12.7	-	-	12	13.4	80	4	0.9	0	-	3	-	-	0	-	-	-	-
16	common ALL	U	F	7	-	4.60	55%	8.40	-	87.2	84.3	86.8	53.3	84	12.9	-	-	9.8	14.9	-	13.7	0.5	8.5	-	-	-	-	-	-	-	-	-
17	common ALL	M	F	1	96%	0.80	16%	4.70	-	99	94.1	92.6	22.9	98.1	1.9	1.3	1.1	3.8	4	54.5	1.1	0.8	5.6	0.7		13.2	1.2	97.9	90.9	5.4		-
18	common ALL	M	F	16	96%	0.80	33%	2.30	IR	99.4	99.2	99	93.9	99.1	1	1.1	3.4	3.4	2.5	4	52.8	0.4	5.1	0	-	0.9	8.3	68.3	95	1.2	-	CD 13
19	common ALL	M	M	11	-	7.20	71%	10.10	-	95.5	88.7	92.9	22.6	92.8	6.9	-	6.9	7.6	8.1	-	38	0.7	3.4	0.4	-	5	-	-	-	-	-	CD13
20	common ALL	M	M	5	100%	19.80	90%	22.00	IR	98.3	99.2	97	46.6	99.6	0.6	1	3.4	1.5	1.9	28.5	32.2	0.7	1.3	0	-	0.2	2.9	25.6	90.5	0.4	-	CD13
21	common ALL	M	M	14	83%	7.60	68%	11.20	IR	98.2	34.7	93.5	6.9	95.4	0.3	0.5	0.1	1.3	0.5	33.9	38.1	0.3	90.1	0	-	1.4	0.6	97.5	84.3	5.3		CD13/ CD33
22	common ALL	M	F	12	96%	0.50	14%	3.80	HR	96.5	99	97.9	23.5	99.7	0.2	0.9	9.4	1.1	1.2	4.7	92.1	1.8	90.2	1.2	11.6	1.3	3.4	91.8	87.8	1.1		CD13/ CD33
23	common ALL	M	M	3	62%	0.10	2%	4.30	SR	96.1	95.2	93.3	7.1	94.9	2.1	0.9	1.1	5.2	1.7	66	1.6	1.5	30.3	1.8	-	11.5	6.2	96.1	88.2	2		CD33
24	common ALL	U	F	6	83%	10.40	73%	14.20	IR	97.9	97.8	86.7	2.2	98.7	1.5	0.4	7.6	1.7	1	55	2.1	0.7	55.1	1.8		3	1.3	56.7	88.7	6.9		CD33
25	common ALL	M	M	10	87%	17.50	64%	27.30	IR	95.6	98.2	94.5	62.5	86.5	0.7	0.7	7.4	0.7	0.6	87.7	2.1	0.4	49.5	0.7		1.8	0.8	99.3	86.3	1.1		CD33
26	common ALL	U	M	4	90%	0.20	5%	3.96	-	100	100	88	0	41	10	-	-	8	0	-	18	-	0	-	-	30	-	-	-	-	-	cyto IgM weakly positive
27	common ALL	M	M	17	100%	111.10	95%	116.90	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	common ALL	U	M	7	90%	2.20	62%	3.50	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	common ALL	U	M	8	95%	27.00	82%	32.90	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	common ALL	M	F	5	95%	6.30	49%	12.90	SR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	common ALL	U	F	2	94%	231.80	95%	244.00	IR	99.9	99.8	95.3	2	96.3	0.2	0.4	1.5	0.6	1.3	65.4	3.3	0.5	33.5	0.2		0.5	0.7	74.5	89.1	3		
32	common ALL	U	M	16	99%	2.80	55%	5.00	HR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	common ALL	U	M	3	90%	21.60	63%	34.20	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	common ALL	M	M	5	90%	31.80	86%	37.00	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	common ALL	M	F	15	93%	13.80	72%	19.20	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	early B precusor ALL	M	F	9	95%	7.10	42%	17.00	-	28.1	2.1	87.8	16	95.9	1	-	-	3.3	3.3	-	1	0.1	0.3	-	-	-	-	-	-	-	-	-
37	early B precusor ALL	M	M	6	90%	0.27	6%	4.50	-	95.6	11.3	88.8	3.5	87.2	23.4	-	-	12.4	12.1	-	0.7	1.3	2.4	-	7	-	-	-	-	-	-	-
38	early B precusor ALL	M	M	4	80%	-	48%	20.90	-	99.7	11.9	98.1	5.2	97.5	3.4	4	0.3	4.3	6.3	61.8	32.7	1.8	15	0.3	-	-	2.8	95.5	54.8	60.5	-	CD13/CD15
39	early B precusor ALL	M	M	1	87%	12.10	28%	43.20	HR	98.8	4.5	93.6	3.1	66	2.5	3.2	0.2	2.7	3.5	44	47.6	1.2	8.8	2.3	-	1.8	4.1	96.1	93.9	97.6		CD15/CD15
40	early B precusor ALL	M	M	5	90%	87.40	84%	104.00	-	34.6	0.2	97.4	0.6	84.4	1	-	-	1.8	1.8	-	8.6	0	13.2	-	-	-	-	-	-	-	-	-
41	pre B-ALL	U	F	1	73%	9.20	47%	19.60	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	pre B-ALL	M	F	3	99%	1.30	13%	10.20	SR	94	89.8	89.6	6.5	99.4	1	0.6	2.5	1.9	0.6	44.1	10.56	0.5	0.7	0.7	-	38.7	0.8	0.8	88.4	0.7		-
43	pre B-ALL	M	F	6	-	7.16	40%	17.90	-	96.3	93	94.6	33.4	94.1	5	-	-	5.6	5.5	-	19.8	0.4	5.4	-	-	-	-	-	-	-	-	-
44	pre B-ALL	U	F	2	92%	-	-	-	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	pre B-ALL	M	F	12	90%	208.80	90%	232.00	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	pre B-ALL	U	M	6	95%	15.70	77%	20.40	-	97.8	93.7	94.9	4.2	93.2	5.4	-	-	5.5	6.3	-	10.4	0.4	6.7	-	-	100	-	-	-	-	-	-
47	pre B-ALL	U	M	4	93%	15.30	75%	20.40	IR	98.4	99.3	96.1	4.2	88.4	0.1	0.1	2.6	0.5	0.5	58.4	16.2	0.2	1.1	0	-	77.5	0.4	16.8	95.8	0.6		-
48	pre B-ALL	U	F	7	92%	0.50	10%	4.90	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49	pre B-ALL	M	F	5	94%	8.10	79%	10.30	SR	95.7	86.3	99.8	3.2	98.6	17	2.8	2.9	3.3	2.1	positive	4.6	0.2	12.1	0.6		56.1	0.8	99.4	98.9	0.5		-
50	pre B-ALL	U	F	8	91%	2.50	35%	7.10	IR	85.2	94.3	94.2	37.4	98.7	1.9	1.6	6.4	2.7	1.8	8	7.2	1.9	0.8	1.1		85.2	1.4	2.1	87.7	5.2		-
51	pre B-ALL	U	F	3	80%	1.10	10%	11.40	-	91.1	90.4	90	4.3	89.1	12.2	-	-	11	9.8	-	43.6	0.7	11.2	-	-	-	-	-	-	-	-	CD13
52	pre B-ALL	M	F	3	90%	5.30	19%	27.90	-	97.3	90.2	94.2	16.5	92.3	5.4	-	-	6.6	11.8	-	52.7	0.5	5.2	-	-	-	-	-	-	-	-	CD13
53	pre B-ALL	M	M	4	65%	21.40	51%	42.00	IR	95.9	98.1	97.5	44.6	96.9	0.5	0.7		0.7	1.7	37.9	6.8	0.4	1.1	0.5		45.9	1.3	0.8	70.7	4.7		
54	pre B-ALL	M	M	2	80%	11.50	64%	18.00	SR	99.6	99.5	96.5	2.1	99	1.5	1.3	5.3	1.8	0.8	53.9	3.7	1.4	8.2	0.2		31.2	2.2	39.3	94.9	3.5		
55	pre B-ALL	M	M	16	99%	0.30	8%	3.50	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
56	pre B-ALL	M	F	10	95%	27.80	74%	37.50	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
57	pre B-ALL	M	M	2	95%	1.10	13%	8.60	SR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
58	precusor T	M	M	5	82%	145.60	78%	186.70	HR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
59	precusor T	M	M	16	83%	486.90	94%	518.00	HR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
60	pro-B	M	M	14	89%	87.20	89%	98.00	IR	99.7	22.5	46.2	11.5	69.5	3.9	1.5	1.8	20.5	2.1	0.1	10.4	42.9	86.9	0.1	-	negative	3.9	26.6	45	9.8		-
61	T-ALL	M	F	8	100%	16.50	73%	22.60	HR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62	T-ALL	M	M	14	91%	105.10	86%	122.20	IR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
63	T-ALL	M	M	12	80%	45.20	59%	76.60	-	-	-	6.5	1.2	2	99.1	-	-	98.8	99.2	-	17.5	0.4	13.9	-	-	-	-	-	-	-	-	-
64	T-ALL	M	M	16	94%	-	45%	25.90	IR	6.9	2.8	0.6	1.9	4.8	99.8	85.1	92.2	99.7	99.7	9.8	73.2	0.4	2.4	0.9	-	0.5	1.9	0.4	3.8	4.8		CD13